Hillard M. Lazarus
Alvin H. Schmaier
Editors

Concise Guide to Hematology

Second Edition

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Dr. Lazarus dedicates this textbook to his loving wife (Joan) of more than 40 years, to their sons and spouses (Jeffrey and Jana; Adam and Sarah), and to their grandchildren (Jacob, Jillian, Aaron, and Benny).

Dr. Schmaier dedicates this book to Linda and to the next generation of physicians in his family Alec, Lauren, Eleanor, and Stella.

Preface

The second edition of our textbook will provide easy-access and up-to-date information in the complex, diverse, and rapidly changing field of hematology and hematologic malignancy. We target entry-level postdoctoral fellows in hematology who are initiating their training as well as medical students and medical interns and residents, clinical nurse practitioners, and physician assistants working in the field of hematology-oncology. We also believe our text will be of considerable use to medical technologists, pharmacy personnel, and pharmaceutical representatives and medical science liaisons. Finally, this book will be attractive to persons working in blood banking and transfusion medicine as well as personnel in cardiology, pathology, infectious disease, and general pediatrics.

The text is presented in an easy-to-read, textual outline format to facilitate rapid and clear information in an organized fashion. This approach differs from other products and allows the reader to locate sections of immediate interest without wading through entire paragraphs to obtain the desired data. Further, the electronic version can be accessed with an "APP." For optimal effect, we utilize a robust indexing tool.

Each chapter is written by a recognized expert(s). The diverse range of authors adds richness on the extent of the progress made in the respective disciplines. The contributors discuss the disorder/disease entity, beginning from incidence/prevalence, to include pathophysiology and patient demographics. Patient-, disease-, and treatment-related issues are discussed.

The reader is provided with up-to-date information but also is given insight and the tools to be able to continue the study of these topics as they develop over time. It is our intent to make this volume the first choice for information for the neophyte who enters the field of hematology.

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Introduction to Hematology

1

Alvin H. Schmaier

Introduction

Hematology is the study of the normal and pathologic aspects of blood and blood elements. Blood is a very unique fluid composed of many cellular elements as well as a liquid portion consisting of proteins, amino acids, carbohydrates, lipids, and other macromolecules and low-molecular-weight precursors. The hematopoietic system is characterized by high cell turnover and replenishment throughout one's life. The pluripotent hematopoietic stem cell (HSC) is the progenitor for all cells that arise in blood. The cellular elements that arise from this stem cell include red blood cells, white blood cells, and platelets. Normal white blood cells in the peripheral circulation include neutrophils, monocytes, eosinophils, basophils, and lymphocytes. Since the HSC also gives rise to cells of the lymphoid system, the study of hematology also includes the lymph nodes and lymphoid tissue. There is no specific organ for hematologic disorders, and its diseases arise within the bone marrow, the lymph nodes, or the intravascular compartment. The latter includes the endothelial cells lining blood vessels and the proteins in the blood plasma. The circulating cell-endothelial cell interface and the rheologic aspects of blood coursing through the intravascular compartment also influence "hematology" and its many parts.

This text has been structured to introduce the trainee to the area of hematology. It should serve as a current introduction to the field of hematology for new fellows. Since the vast majority of medical students and residents do not become hematologists, there are certain essential items that all trainees must learn about this area of medicine. Using this text, the trainee will learn the physician's approach to anemia and red blood cell disorders and be able to fully evaluate a complete blood count (CBC). Screening tests for bleeding disorders for the diagnosis of an individual who has a defect in the proteins or cellular elements that prevent bleeding will be described. The trainee also will be exposed to the clinical, biologic, and genetic risk factors that contribute to thrombosis. Finally, the student will be introduced to those white cell disorders that are diagnosed and treated by non-hematologists and the uncommon but serious neoplastic white blood cell disorders where a hematology consultation is needed.

Origins of Hematopoietic Cells

Hematopoiesis begins early in embryonic development. The HSC and the blood vessel lining cells or endothelial cells are thought to be derived from the same precursor cell in the aorta-gonad-mesonephros (AGM) system. The common precursor to the HSC and the endothelial cell is the hematoblast. It has been proposed that this cell has the capacity to differentiate into both cell classes. The HSC is present in very small numbers and retains its ability to differentiate into all blood cells as well as proliferate. In the earliest stages of embryogenesis, these cells circulate through the embryo to supply oxygen and deliver nutrients. The stem cells that arise from the AGM later in embryogenesis give rise to the blood system starting initially in the yolk sac and then seeding the liver and finally the bone marrow. These cells demonstrate the ability to "travel" from the time they leave the yolk sac to populate tissues and still circulate in small numbers even in adults, a property exploited in hematopoietic cell transplantation. These cells regress in the liver, kidney, and spleen, but in times of stress, they can resume blood product production as seen in myeloproliferative disorders and myelofibrosis. Blood production is under very tight control in order to maintain the proper number and ratio of blood cells. Specific growth and transcription factors regulate cells to become committed to specific lineages.

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The Myeloid System

Cells of this group arise in the central marrow cavity (called the "medullary" cavity). Myeloid lineage blood cells arising elsewhere in the body are designated as "extramedullary" in origin. The myeloid system consists of the following cells: red blood cells (erythrocytes), white blood cells (neutrophils, monocytes, eosinophils, basophils), and platelets (thrombocytes). Neutrophils, eosinophils, and basophils have been collectively called "granulocytes" because the presence and nature of their cytoplasmic granules define their function; however, when physicians use the term "granulocytes," they are often referring to just neutrophils.

1. *Erythrocytes* (red blood cell, RBC): An erythrocyte is a specialized anucleated cell that packages hemoglobin, the protein that is a respiratory gas transport vehicle that carries oxygen from the lungs to tissues and carries carbon dioxide from tissues back to the lungs to be dispelled. Erythrocytes undergo erythropoiesis whereby they mature from the myeloid progenitor cell to the nonnucleated, highly deformable biconcave disk approximately 8–10 μ in diameter. The absence of a nucleus and the very flexible cell membrane confers the ability to bend and to traverse 2–3 μ capillaries. Red blood cell production is regulated by the hematopoietic growth factor, erythropoietin. The process of erythropoiesis takes 4 days to produce a nonnucleated biconcave disk that enters the circulation with residual RNA in its cytoplasm. A new RBC in the circulation, termed a reticulocyte, is slightly bigger than older cells. The reticulocyte count is identified by the use of a special stain that represents the percentage of early RBC compared to the total number of RBC in the circulation. Red blood cell RNA remains in the erythrocyte about 1 day, so a normal "reticulocyte count" is <2%. The red cell life span is 120 days, and normally there are about 5 million RBC/μl in whole blood in adult males and 4.5 million RBC/μl in adult females. Old RBCs lose their energy-producing (ATP) capacity, develop stiff cell membranes, and are removed from the circulation by the macrophages of the mononuclear-phagocytic system of the spleen. Their hemoglobin is normally retained in the reticuloendothelial (RE) system but can be lost when there is brisk, shortened red blood cell survival, i.e., hemolysis.
2. *Neutrophils* also are referred to as polymorphonuclear neutrophils, PMN or polys, segmented neutrophils, or segs, the name derived from the nucleus that is usually a three- to four-lobed or "segmented" structure that stains a bluish color with Wright-Giemsa stain. An early form of a neutrophil is a "band" that shows an unsegmented nucleus. A neutrophil normally takes 12–13 days to be produced in bone marrow. Its life span in the circulation is about 12 h, and they can live in tissues for several days. The marrow pool of mature neutrophils is 30–40 times that seen in the circulation. Outside the marrow, half of the neutrophils are "marginated" or adherent to the endothelial cells. Margination of neutrophils allows them to serve as a "reserve" to be quickly released in times of stress such as infection. Only one half of the neutrophils that circulate are reflected in the "white blood cell (WBC) count." In the adult, neutrophils constitute 50–80% of the total WBC analyzed (4000–10,000/μl). Neutrophils exit the circulation via diapedesis into tissue through the capillary junctions in response to chemotactic stimuli. Their functions are to phagocytize and digest bacteria, cellular debris, and dead tissue. Both neutrophils and monocytes are part of the body's innate immunity in contrast to adaptive or learned immunity of lymphocytes (see below).
3. *Monocytes* are large, mononuclear cells with an indented (kidney-shaped) nucleus that form the circulating component of the mononuclear phagocyte system. The nucleolus in mature monocytes circulating in the peripheral circulation is usually not identified in blood by light microscopy. Monocytes spend 1–3 days in bone marrow and 8–72 h in the peripheral blood. They have a similar functional role to neutrophils in host defense against organisms. Once they traverse into tissues, they can differentiate into *macrophages* that can survive in tissues for long periods (up to 80 days). Macrophages are tissue-resident as opposed to circulating monocytes. Macrophages are characterized and named for their tissue of origin: alveolar macrophages in the lung, Kupffer cells in the liver, splenic macrophages, and oligodendrocytes/glial cells in the brain. They function to phagocytize pathogens, cellular debris, and dead tissue.
4. *Eosinophils*: Eosinophils are characterized by their prominent orange-reddish [refractile] granules seen on Wright-Giemsa stain. Eosinophils usually have bilobed nuclei. Eosinophils increase in reaction to foreign protein and thus are seen in parasitic infection (especially larva of roundworms, helminths), allergic conditions, cancer, and certain drugs. Granules contain several proteins, most notably major basic protein (MBP). Normally eosinophils constitute 0–2% of the WBC differential cell count.
5. *Basophils*: Basophils are equally colorful with very dark, bluish prominent granules following Wright-Giemsa stain. Granules contain histamine, heparin, and hyaluronic acid. Histamine release (basophil degranulation) is part of the allergic reaction. Normally basophils are 0–1% of WBC differential blood count. They are often increased in patients with chronic myeloid leukemia and other myeloproliferative disorders. Mast cells arise from separate bone marrow precursor cells than basophils and have prominent granules that have a role in host defenses against parasites.
6. *Platelets* (thrombocytes): Platelets bud off from the cytoplasm of the bone marrow megakaryocytes. The "mega"

karyocyte in the bone marrow is recognized by its large size. Uniquely, the cell doubles its nuclear and cytoplasmic material but does not divide. Megakaryocyte growth and platelet segmentation is regulated by the hematopoietic growth factor thrombopoietin. Platelets are anucleated cell fragments that contain remnant mRNA. They have a 7–10-day life span, and their first 1–2 days are spent in the spleen. Platelets may be entrapped by an enlarged spleen as seen in congestive and inflammatory disorders; the resulting hypersplenism may result in thrombocytopenia. They have a central role in hemostasis as they contain many hemostatic cofactors and inhibitors in their granules. They also have a role in inflammation since they contain many growth factors. At the megakaryocyte level, plasma proteins can be adsorbed and packaged into platelet granules.

Mononuclear Phagocytic System

The mononuclear phagocyte system consists of circulating monocytes derived from the myeloid progenitor cells in the bone marrow that migrate from the circulation into tissues and differentiate into macrophages. The mononuclear phagocytic system is also called the reticuloendothelial (RE) system. These cells are found in the bone marrow, thymus, lymph nodes, spleen, serosal surfaces, adrenal cortex, Peyer's patches, and Waldeyer's ring. They function as a "cleanup system" for circulating debris, microorganisms, and aged, defective, or antibody-coated RBC.

Lymphocyte System

Lymphocytes reside mostly in lymph nodes, but also large numbers are detected in blood and bone marrow components. As already mentioned above, they are part of our adaptive immunity system. The major lymphocyte subsets are B and T cells. NK (natural killer) cells are a specialized lymphoid population. All cells arise in the bone marrow, but T cells mature in the thymus, and B cells mature in the lymph nodes, spleen, or other lymphoid tissues, e.g., Peyer's patches in the gut and Waldeyer's ring in the throat. Immunosurface markers are used to classify lymphocytes. B cells are identified by CD19 and CD20. T cells are identified by CD3, CD4, or CD8. NK cells comprise 10% of circulating lymphocytes and are identified by the CD3–CD56+ phenotype.

The Physical States of Blood

(A) Blood is a *suspension* of cells in a solute of water, water-soluble proteins, and electrolytes.

(B) The *viscosity* of blood = 1.1–1.2 centipoise. The viscosity of blood is highly influenced by red blood cell and protein concentration. Increased viscosity can occur from an elevation in the cellular components as is seen in polycythemia (increased numbers of red blood cells) and protein as seen in disorders such as multiple myeloma (elevated IgG levels) and Waldenström's macroglobulinemia (elevated IgM levels). Red cell size (smaller size increases viscosity as cells are less deformable) and the speed of blood flow in a given vessel also influence viscosity (viscosity in the aorta is much less than in a small arteriole). High elevations of myeloid cells as in certain forms of acute leukemia and myeloproliferative neoplasms also influence blood viscosity.

(C) Blood volume averages 70 ml/kg of body weight; thus the 70 kg adult has roughly 5 l of blood. The blood volume of an individual (man, dog, etc.) is approximately 7% of the total body weight. Children may have a slightly higher % (~10%) blood volume to total body weight.

(D) Cellular composition of blood averages 38–42% in women, 40–44% in men; the percent volume contributed by red blood cells is called the *hematocrit* or packed cell volume.

(E) *Plasma* is anticoagulated blood (i.e., blood where the calcium chloride has been chelated, i.e., bound and not available for interaction with proteins) from which the cellular components (red cells, white cells, and platelets) have been removed by centrifugation. It contains the blood coagulation proteins. *Serum* is the liquid in blood remaining after clot formation from a blood sample that has been collected without an anticoagulant. Many of the blood coagulation proteins have "clotted" and formed a precipitate along with the cellular components of the blood. In this process, the cellular components, platelets, neutrophils, etc. become activated and release their granule contents. It is usually yellow in color unless the red blood cells are lysed (hemolyzed) releasing free hemoglobin that gives a red color in visible light. Serum is prepared from a "red" top tube.

Plasma coagulation studies can only be performed on blood that was obtained with a proper anticoagulant (usually sodium citrate in clinical medicine) and the plasma separated from the blood cells. Sodium citrate is the so-called "blue-top" tube. Characterization of the proteins of hemostasis and thrombosis is performed in the artificial environment of collection of whole blood in sodium citrate.

Blood to be collected for CBC and differential blood count are collected in the anticoagulant EDTA (ethylenediamine triacetic acid). It is collected in the so-called "lavender-top" tube. Plasma prepared from EDTA anticoagulation is invalid to evaluate the blood coagulation system.

Hematopoiesis

2

Gabriel Ghiaur and Richard J. Jones

Introduction

Hematopoiesis (hemato, blood; poiesis, to make) is a highly organized process that results in the generation of all cellular elements of blood to serve oxygen delivery needs, coagulation, and immune host defenses. Complex mechanisms regulate hematopoiesis not only in order to maintain homeostasis (generation of new elements to replace dying cells) but also to adapt to physiological stress and to do so for the entire life of an organism. Moreover, because the half-life of blood cells is short ranging from hours (granulocytes) to days [red blood cells (RBCs) and platelets], the physiologic requirements of hematopoiesis must generate billions of blood cells daily. Hematopoiesis is capable of achieving these goals and maintains homeostasis due to its unique characteristics: distinct ontogeny and physiology, complex anatomy with medullary (red marrow of axial bones) and extramedullary organs (spleen, liver, thymus, lymph nodes), hierarchical structure that follows an orderly differentiation from stem to progenitor to precursor to mature cells, and tight regulatory networks that match the physiological demands with mature cell output. From a clinician standpoint, hematopoiesis is most frequently interrogated at the level of mature blood cells. Nevertheless, various aspects of this process are important to be understood as they occasionally have profound clinical implications.

Hematopoietic Ontogeny

Primitive Hematopoiesis

The first hematopoietic cells found in the developing embryo are primitive RBCs that are produced in the yolk sac [1–3]. RBCs contain hemoglobin to match the increased oxygen requirements of the developing embryo; fetal hemoglobin has unique binding affinities that allow oxygen to be carried from the placenta to even more hypoxic places in the growing embryo. The initial wave of hematopoiesis is restricted to address this particular need, generating primitive fetal nucleated RBCs and only a limited variety of other cells such as iron delivering monocytes to help with the hemoglobinization process.

Definitive Hematopoiesis

During mid-gestation, the liver becomes the major hematopoietic organ [2, 4], playing a similar role to the bone marrow during adult life. This embryonic period is the primary time during development when true hematopoietic stem cell expansion occurs. Nevertheless, with the development of axial skeleton, the spleen, and the thymus, hematopoiesis migrates out of the liver and populates the "adult" hematopoietic organs around birth. Migration into the bone marrow, in particular, is an active process toward a CXCL12 (SDF1α) gradient and depends on CXCR4 receptor on hematopoietic stem and progenitor cells which binds to CXCL12. To this end, genetically engineered mice lacking either CXCR4 or CXCL12 have normal hematopoiesis in the fetal liver stage but have near absence of hematopoiesis in the bone marrow [5, 6].

Adult Hematopoiesis

After birth, hematopoiesis takes place mostly in the bone marrow. Additional lymphohematopoietic organs such as spleen, thymus, and lymph nodes have specialized functions in the development of subsets of lymphoid cells. Hematopoiesis in the bone marrow occurs mostly in the axial bones (vertebrae, pelvic and scapular bones, skull, and sternum). Total bone marrow cellularity changes with age such that hematopoietic cells occupy over 90% of total marrow

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space at birth but only about 20% in older-aged individuals. Except for extreme ages, using the formula "100 – age" generally gives a good estimate of expected bone marrow cellularity (e.g., a 30-year-old marrow should exhibit about 70% cellularity). Lymph nodes and thymus represent unique environments, whose main functions are the terminal differentiation and education of B and T lymphocytes, respectively. The thymus reaches its peak activity during puberty and then undergoes involution with only remnant tissues present in advanced-aged individuals.

Clinical Implications of Hematopoietic Ontogeny

Hemoglobin Switches During Embryonic Development and Its Role in Sickle Cell Disease

As mentioned above, one of the main functions of the hematopoietic system is to ensure oxygen delivery. To this end, during ontogeny there are two main hemoglobin switches that optimize the oxygen-delivering capacity of RBCs for the physiological conditions during that period of time. Primitive RBCs produced in the yolk sac contain embryonic hemoglobin, perfectly suited to deliver oxygen in the relatively small embryo. As the fetus develops, fetal hemoglobin (Hg F) present in definitive RBCs produced in the fetal liver has high affinity for oxygen to upload it from maternal Hg A1 in the highly vascular placenta and carry it to the increasingly more complex embryo. The third and most important switch in hemoglobin happens around birth and results in replacement of Hg F with Hg A1 that has lower affinity for oxygen so it can optimally carry it from the lungs and release it in the peripheral tissues [7]. These changes are the results of complex control of beta-globin gene. Though usually not reversible, these switches can also occur in some pathological conditions or during treatment with certain drugs. For instance, in patients with beta hemoglobinopathies such as sickle cell disease, treatment with hydroxyurea may result in epigenetic changes that allow for increased Hg F and lower levels of the pathogenic Hg S, potentially alleviating some of the disease's morbidity [8].

Myelophthisis (Displacement of Hematopoietic Bone Marrow: Phthisis – Shrinkage or Atrophy) and Extramedullary Hematopoiesis

Generally most myelopoiesis takes place in the bone marrow after birth. However, conditions in which the bone marrow is occupied by other processes such as hematologic malignancies or solid tumors metastatic to the bone turn the microenvironment inhospitable or insufficient to accommodate normal hematopoiesis. In these situations, hematopoiesis can find home in secondary hematopoietic organs such as the spleen, lymph nodes, or liver [9, 10]. This process produces myelophthisic anemia, and a peripheral blood smear could identify a variety of young hematopoietic elements in various stages of differentiation creating a so-called "leucoerythroblastic" pattern.

Hierarchical Organization (Fig. 2.1)

Hematopoietic Stem Cells

Hematopoiesis has a hierarchical structure that starts with the hematopoietic stem cell (HSC) compartment. The HSC compartment includes a variety of cells that are functionally defined by two characteristics – multipotential differentiation and self-renewal – that are required to maintain the blood system for the lifetime of the host. The extensive proliferative capacity of HSCs is best exemplified during bone marrow transplantation (BMT) when donor HSCs give rise to a complete hematopoietic system in the new host; of note, *one HSC has the ability to fully repopulate the entire mouse blood system* [11]. The self-renewal properties of HSCs imply that upon division, a HSC gives rise to at least one daughter cell that maintains its characteristics. Though this property has been demonstrated in depth in murine models via serial BMT of limiting numbers of HSCs [12] and it is likely attributable to human HSCs as well [13], formal proof of self-renewal of human HSCs is still lacking. Some of the cells within the HSC compartment, the so-called long-term or high-quality HSCs, are generally quiescent, contributing to their high resistance to chemotherapy and radiation therapy. These high-quality HSCs are rarely recruited into cell cycle during steady-state hematopoiesis or even during minor hematopoietic stressors, but they do respond to major hematopoietic stress (for instance, during recovery from chemotherapy or after BMT). Other slightly more differentiated stem cells that have less capacity for extensive self-renewal but can still maintain hematopoiesis for periods of time ranging from months to years could be considered low-quality HSCs [14]. Such low-quality HSCs are responsible for splenic colonies (CFU-S), as well as the waves of hematopoiesis, seen after transplantation of human HSCs in immunodeficient mice [14]. Human HSCs (probably only low-quality ones) can be functionally assessed by transplantation into immunodeficient or SCID mice, the so-called SCID-repopulating capacity (SRC) [13]. There is also accumulating evidence that mutations in high-quality HSCs result in poor prognosis AML (i.e., AML from MDS), while mutations in low-quality HSCs may result in "good" prognosis AML (i.e., APL or CBF AML) [15, 16].

The gold standard definition of HSCs implies functional characterization of these cells, i.e., repopulation of the entire blood system; accordingly, this event can only be accomplished in animal models. Thus, a phenotypic characterization

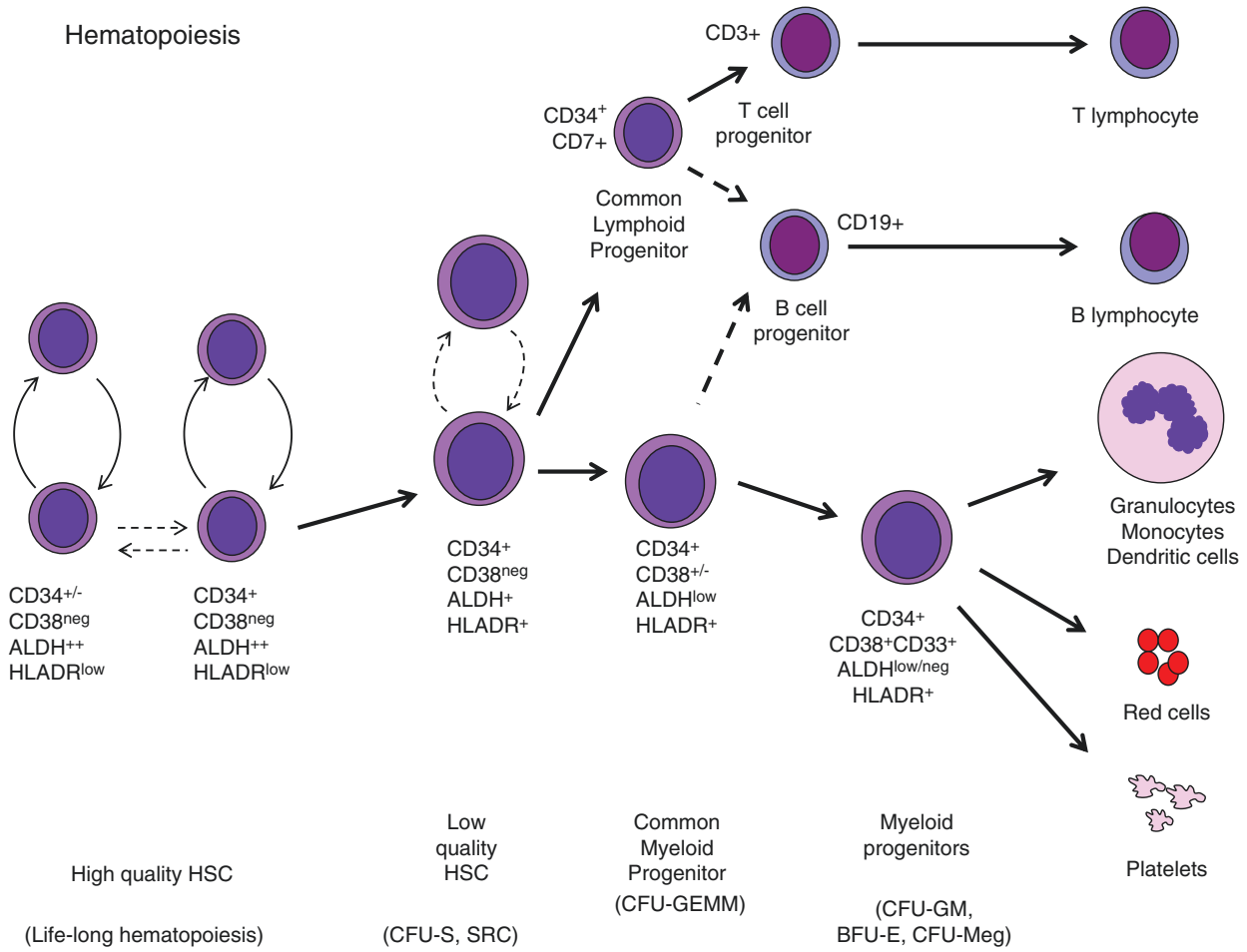

Fig. 2.1 Human hematopoiesis. Hematopoietic cell lineages are based on a hierarchical system. The hematopoietic stem cell can self-renew and differentiate into progenitors that can give rise to all blood cell types. HSC hematopoietic stem cell; ALDH aldehyde dehydrogenase; CFU-S spleen colony-forming unit; SRC SCID mouse repopulating capacity; CFU-GEMM granulocyte, erythroid, monocyte, megakaryocyte colony-forming unit; CFU-GM granulocyte colony-forming unit; BFU-E erythroid blast-forming unit; CFU-meg megakaryocyte colony-forming unit

would have many potential advantages, especially for ease of study in humans and prospective isolation for clinical use. However, there is no combination of markers that clearly distinguishes human high-quality from low-quality HSCs and HSCs from their more differentiated progenitor cells (HPCs); these cells represent a continuum where they gradually lose self-renewal capacity as they differentiate. Nevertheless, there is general agreement that high-quality HSCs express CD34 (a marker expressed also by HPCs as well as endothelial cells) and have high levels of aldehyde dehydrogenase (ALDH, an enzyme important in retinoic acid biosynthesis which is required for their growth and differentiation). In addition to high ALDH activity, HSCs exhibit a relative lack of blood differentiation markers like CD38 and expression of CD90. As high-quality HSCs differentiate into low-quality HSCs and then HPCs, they gradually lose CD34 and ALDH expression while gain expression of CD38 and other differentiation markers.

HSCs are relatively resistant to most chemotherapy agents. Not only are HSCs quiescent, thus displaying kinetic resistance, but they upregulate most drug-detoxifying enzymes as well as ATP-binding cassette (ABC) transporters that can actively pump out toxic drugs from the cells [17]. HSCs also reside in a highly protective microenvironment or niche, which has the ability to inactivate cytotoxins in part through high expression of cytochrome P450 enzymes [13, 18, 19]. ALDH, also known as retinaldehyde dehydrogenase, the rate-limiting step in the metabolic activation of vitamin A to retinoic acid, is responsible for HSC resistance to cyclophosphamide [20]. ALDH actually inactivates cyclophosphamide by serendipity, through oxidation of the active metabolic aldehyde intermediate aldophosphamide to the inactive carboxylic acid carboxyphosphamide. HSC resistance to cyclophosphamide allows the drug to be given at high doses after allogeneic BMT as graft-versus-host disease (GVHD) prophylaxis; this advance now allows successful

partially mismatched allogeneic BMT (see Chap. 36 on "Hematopoietic Cell Transplantation") [21].

Many leukemias appear to originate from the HSC compartment, including myeloproliferative neoplasms (MPN) such as chronic myeloid leukemias, essential thrombocythemia (ET), polycythemia rubra vera, and myelofibrosis, myelodysplastic syndromes (MDS), and some acute myeloid leukemias (AMLs) [22, 23]. Recent data suggest that unfavorable AMLs often arise from high-quality HSCs. Leukemias arising from high-quality HSCs generally are also relatively drug resistant, at least in part by co-opting normal HSC drug resistance mechanisms [16]. AMLs with more favorable prognoses appear to arise from lower-quality HSCs and progenitors [15, 16].

Hematopoietic Progenitor Compartment (HPCs)

The progenitor compartment is one of the most diverse cellular compartments in the human body; it is comprised of multiple cell types with various differentiation and self-renewal potential. In general, there is more and more restricted self-renewal and differentiation potential as the progenitor cells mature toward the precursor compartment. The strict definition of HPCs relies on their ability to form colonies in semisolid media. Thus, they are also called colony-forming units (CFUs) [24]. A colony (at least 50 cells), as opposed to a cluster (generally 20 cells or less), has to be able to undergo at least 5 divisions ($2^5 = 32$) before becoming mature, postmitotic cells. These cells are most responsible for maintaining hematopoiesis during times of stress. Because HPCs are highly proliferative, they are relatively sensitive to chemotherapy. These cells continue to express the HSC marker CD34, as well as differentiation markers such as CD38. In addition, specific lineage markers differentiate between myeloid progenitor cells (CD33 and HLA-DR) and lymphoid progenitors (the T-cell marker CD3 or the B-cell marker CD19).

The Precursor Compartment

The precursor cells are lineage-committed hematopoietic cells that still have some proliferation potential but are mostly undergoing maturation and induction of terminal differential as they generate the mature elements of blood. These cells are identified in the bone marrow by specific morphological features or through specialized cell staining methodology. The first identifiable myeloid precursors are the promyelocytes, which appear as large cells with multiple coarse granules that cover the entire cytoplasm and nucleus. These cells will further divide and acquire more cytoplasm, and the nucleus will become indented as it matures to myelocytes, metamyelocytes, bands, and finally polymorphonuclear neutrophils. The nuclear indentation is a sign of chromatin inactivation and will result in nuclear segmentation when maturation is complete with the emergence of mature polymorphonuclear neutrophil. This process is influenced by various hematopoietic stressors such as infections or recovering from chemotherapy, which will increase the number of immature precursors in the marrow and blood. The process is also blocked by autoimmune reactions, vitamin deficiencies, or malignancies in the marrow.

Erythroid differentiation is characterized by parallel maturation of the cytoplasm, i.e., acquisition of hemoglobin, and of the nucleus, i.e., condensation of chromatin, until the nucleus becomes pyknotic and is eventually expelled. Processes associated with accelerated production of RBCs, such as ineffective erythropoiesis (MDS or folate/B12 deficiency) or destructive processes (chronic autoimmune hemolytic anemias, bleeding) disrupt this balance and result in megaloblastic/megaloblastoid changes in the erythroid compartment. Megaloblastic anemia refers to abnormal synchrony in the nuclear and cytoplasmic differentiation seen in folate and in B12 deficiency, such that the cytoplasm matures faster than the nucleus. Megaloblastoid changes resemble this process but are generally associated with other red cell morphologic changes seen in the setting of MDS. Some viral infections (for instance, parvovirus B19) induce apoptosis of erythroid precursor cells and result in profound anemia. In this case, a bone marrow biopsy would show multiple infected proerythroblasts characterized by intranuclear inclusions resembling nucleoli ("giant proerythroblasts").

A morphologically unique precursor cell is the megakaryocyte. This is a multinucleated cell that is located close to the sinusoid vessels of the bone marrow and "sheds" platelets into circulation, a process resulting from budding of the megakaryocyte cytoplasm. Its characteristic morphology is disrupted in bone marrow disorders resulting in unique forms such as micromegakaryocytes as seen in autoimmune thrombocytopenia, CML, or MDS or staghorn megakaryocytes, abnormally large cells with nuclear forms resembling renal staghorn calculi seen in ET and myelofibrosis.

Mature Blood Cells

The mature blood cell compartment is the part of hematopoiesis that is most often interrogated by the clinician using a simple complete blood count (CBC). Any abnormality present on a CBC with differential is generally an indication for a peripheral blood smear evaluation. The peripheral blood smear can give information about the morphology of all blood lineages as well as some physiological information about the rheology of blood. Mature blood cells serve the three main functions of blood: oxygen delivery (RBCs),

hemostasis (platelets), and host defense – innate immunity (granulocytes and NK cells) and acquired immunity (lymphocytes). Changes in numbers and function of these cells are of utmost importance in maintaining health and will be discussed elsewhere in this book. Several characteristics of mature blood cells (other than number and function) are also particularly important to recognize as they have clinical implications. Most mature blood cells, except for lymphocytes, are fully differentiated and unable to divide further. RBCs survive up to 120 days, platelets 9–14 days, and granulocytes several hours. When transfused, the life spans of these cells are even shorter, and this is reflected in the frequency of transfusions needed by patients with deficiencies of these cells. Moreover, whole blood cell transfusion (rarely used nowadays) or contamination of RBC or platelet products with lymphocytes could result in passive transfer of donor lymphocytes into recipient. For the most part, this process bears no clinical significance as these cells will be eliminated by the host immune system. However, in profoundly immunosuppressed patients, such as newborns and patients recovering from chemotherapy or BMT, passive transfer of lymphocytes can result in severe and often fatal transfusion-associated GvHD. Thus, blood products should be irradiated when given to these patients.

Regulation of Hematopoiesis

The bone marrow microenvironment or niche regulates hematopoietic activity to meet physiological needs in healthy individuals. There are two major components of microenvironment: cellular elements and noncellular elements represented for the most part by growth factors and extracellular matrix.

Bone Marrow Niches

Within the bone marrow, hematopoietic stem and progenitor cells differ in their location and likely occupy different anatomic areas [25]. The classical view is that some of the most quiescent HSCs are located close to the endosteum in bone marrow niches that have low oxygen tension, low pH, and high calcium. Interaction with osteoblasts tethers these cells in the bone marrow and is essential to maintain quiescence. In these niches, HSCs are relatively isolated from the systemic circulation and are maintained in their primitive undifferentiated state. In contrast, endothelial cells create niches that have higher oxygen tension, are easily accessible by circulating factors, and promote HSCs to proliferate and differentiate into HPCs. Constant migration of HSCs between these niches and out of the bone marrow and into the systemic circulation is seen at baseline and especially during hematopoietic stress. Forced egress of HSCs and HPCs out of bone marrow niche into the systemic circulation, a process called mobilization, can be achieved by either chemotherapy [26], treatment with hematopoietic growth factors such as G-CSF [27], or inhibition of the CXCR4-CXCL12 axis [28]. Upon mobilization, HSCs and HPCs can be collected from peripheral blood and used for transplantation.

Growth Factors and Extracellular Matrix

In various niches in the bone marrow, stromal cells produce soluble and membrane-bound growth factors that promote survival, proliferation, and differentiation of HSCs and HPCs. Some of the stromal cells also produce extracellular matrix proteins such as fibronectin, collagen, laminin, and glycosaminoglycans that are important in tethering HSCs as well as some of the growth factors. The HSCs/HPCs express receptors that interact with stroma cells, the extracellular matrix, as well as growth factors. As mentioned earlier, these receptors are essential for colonization of bone marrow with HSCs/HPCs during development or upon BMT. Of these, integrins and the CXCL12-CXCR4 axis play key roles not only in migration to the bone marrow but also in anchoring HSCs in the bone marrow niche [29]. To this end, treatment with plerixafor, a small molecule that blocks binding of CXCL12 to CXCR4 and thus inhibits CXCR4 function, results in loss of anchorage and mobilization of HSCs into circulation [28].

Many growth factors have dual actions on hematopoiesis, with effects both early in hematopoiesis on the more primitive HSCs/HPCs and later on differentiated precursors. Accordingly, three of the most important growth factors for HSCs, kit ligand [30, 31], FLT3 ligand [32], and thrombopoietin (TPO) [33] also play key roles for differentiated precursor cells. Kit ligand, produced by stromal cells, activates the kit receptor and provides anti-apoptotic signals to HSCs and primitive HPCs. The kit receptor is also highly expressed by, and plays a critical role in, mast cells [34]. Similarly, FLT3 ligand binds the FLT3 receptor and promotes proliferation of HSCs/HPCs as well as maturation of dendritic cells [32]. Lastly, TPO produced mostly by the liver binds its receptor cMPL on stem cells and megakaryocytes/platelets [33]. TPO is constantly produced by the liver and cleared from circulation by binding to cMPL on platelets. Thus, high platelet numbers result in lower TPO levels and decreased megakaryopoiesis. Of note, decreased levels seen in acute or chronic liver failures may result in thrombocytopenia.

Similar to TPO, erythropoietin (EPO) is also produced by a non-hematopoietic organ, the kidney. As such, TPO and EPO are actually hematopoietic hormones. EPO binds the EPO receptor and contributes to final maturation of RBCs [35]. The oxygen-sensing machinery in the juxtaglomerular

apparatus of the kidney produces EPO in response to low oxygen delivery via HIF1 complex [36]. EPO acts on the hematopoietic cells in the bone marrow to increase production of RBCs. Renal failure states are associated with impaired EPO production and eventually anemia. Supplementation with recombinant EPO can improve the anemia associated with renal failure.

Although not active on HSCs, both G-CSF and GM-CSF play important roles not only on HPCs but also on terminally differentiated neutrophils and monocytes [37]. Although both can be used clinically to promote neutrophil recovery, in routine patient care, G-CSF and sometimes PEGylated G-CSF are preferred. On the other hand, either hereditary or acquired GM-CSF deficiency results in impaired macrophage function and presents as pulmonary alveolar proteinosis. This condition is due to accumulation of proteinaceous material in the alveolar space, otherwise cleared by the alveolar macrophages. Treatment with GM-CSF significantly improves the clinical outcome of some of these patients [38].

Abnormal Hematopoiesis

The hierarchical structure of hematopoiesis and the multiple mechanisms that control all aspects of this process ensure hematopoietic homeostasis as well as rapid adaptation in response to stress. Most abnormalities in hematopoiesis are characterized by either lack or overproduction of various mature blood elements. More recently, important qualitative abnormalities, such as clonal hematopoiesis, are being increasingly recognized.

Leukopenia

Leukopenia or low number of white blood cells (WBCs) can be the result of low lymphocyte counts (lymphopenia) or low neutrophil counts (neutropenia) (Table 2.1). Because humans are endowed with a high reserve of normal WBCs, substantial decreases in WBC numbers often are associated with no functional sequelae. Moreover, the majority of lymphocytes (which primarily reside in lymphoid organs such as lymph nodes and spleen) and neutrophils (which are marginated to the blood vessel wall and other tissues) are not circulating and will not be counted in the CBC. Accordingly, although the CBCs are usually a reflection of total body WBC pools, this may not always be the case.

Lymphopenia results in decreased adaptive immunity. Constitutive lymphopenias are seen in some patients with mutations in either cytokine receptors of signaling molecules important for lymphogenesis. One such case, severe combined immunodeficiency (SCID) results in near absence of T cells and adaptive immunity [39]. Acquired lymphopenias could be secondary to drugs or viral infections as seen in infection with HIV causing depletion of T lymphocytes or may be secondary to decreased production as seen with decreased numbers of normal B cells and normal immunoglobulins in patient with CLL [40]. The most common immunodeficiency, common variable immunodeficiency, primarily affects B cells and is generally not associated with significant leukopenia since these cells represent a minority of circulating lymphocytes. The universal use of immunoglobulin replacement has lessened clinical implications of this disease of unknown, but probably genetic, origin.

Table 2.1 Classification of leukopenias and neutropenias

Cell type affected	Clinical condition	Clinical consequences	Pathophysiology	Potential therapy
Lymphocytes	SCID	Absence of T cells	Mutations in IL2 Rγ	BMT
		Absence of adaptive immunity		Gene therapy
	Acquired lymphopenias	Depletion of T cells	May be secondary to viral infections such as HIV	Treat HIV
		Decreased production of normal B cells and normal IgG	This may be seen in CLL when the malignant lymphocytes not only don't produce functional Ig but also interfere with the normal lymphopoiesis	My require prophylaxis therapy
				Treat CLL
				May require IVIG
	CVID	Low production of Ig and recurrent infections	May have normal B cells but with impaired function	IVIG replacement
Neutrophils	Constitutional neutropenia	Normal variant	More frequent in some racial populations (i.e., African American)	No need for therapy
	Autoimmune neutropenia	Decreased number of neutrophils and increased risk of infections	Anti-neutrophil antibodies	Abtx prophylaxis
	Cyclical neutropenia	Decreased number of neutrophils is cyclical, sometimes every 21 days	Acquired or Inherited (autosomal dominant mutations in ELANE)	Depending on severity may require GCSF, prophylactic antibiotics, or no intervention
	Drug-induced neutropenia	Profound neutropenia and agranulocytosis with subsequent increased risk of infections	Marrow toxic drugs (i.e., chemotherapy) or idiosyncratic	Remove offending agent, may require GCSF treatment

Neutropenia is generally categorized as mild (counts between 1000 and 1500 per μL), moderate (counts between 500 and 1000 per μL), and severe (counts <500 per μL). However, mild to moderate neutropenia is usually well-tolerated with little increased risk for infection. Such benign neutropenias are more frequent in African Americans (benign ethnic neutropenia) or can be autoimmune in etiology. The differential diagnosis for acquired neutropenia is extensive and, in addition to autoimmunity, can be drug-induced (particularly after cancer chemotherapy) or associated with impaired production of normal neutrophils as seen in MDS or acute leukemia. Though supplementation with G-CSF may fasten neutrophil recovery after chemotherapy, there are no conclusive studies that this improves survival in patients with neutropenia. Granulocyte transfusions are generally not used given the multiple potential complications as well as the relatively short half-life of these cells. Nevertheless, in the face of life-threatening infections (particularly fungal infections), granulocyte transfusions could be used in combination with antifungals temporarily.

Anemia

Anemia is defined as decreased hemoglobin concentration. It is important to recognize that anemia may be associated with decreased, normal, or increased numbers of RBCs. Most anemias are associated with low RBC and reticulocyte numbers including those resulting from relatively low EPO levels as in chronic kidney disease, metabolic deficiencies (B12 and folate), or primary bone marrow disorders (leukemia or MDS). After the cause has been determined and treated, these anemias will show a rapid rise in reticulocytes that predates the normalization of hemoglobin. Other forms of anemias, such as thalassemia or sickle cell disease, may actually be associated with higher number of RBCs; in such cases, the anemia is associated with low hemoglobin concentrations per cell. Although higher transfusion thresholds have been used in the past, transfusion support to maintain a hemoglobin >7 g/dl is now usually the standard.

Thrombocytopenia

Thrombocytopenia is most often not associated with increased risk of spontaneous bleeding until platelet counts are below 50,000 per μL, and often not until less than 20,000 per μL. Patients with immune thrombocytopenia often tolerate platelets of even less than 5000 per μL, as the platelets are very young, larger than usual, and have improved hemostasis. In such patients, a platelet reticulocyte count or immature platelet fraction will usually show a higher proportion of young platelets, suggesting increased destruction as the cause of the thrombocytopenia. Other causes of thrombocytopenia are the same as those conditions that cause granulocytopenia or anemia (see Chapters 4 and 15). Transfusions in patients not producing platelets are generally reserved for platelet counts <10,000 per μL, unless there are associated bleeding events, abnormal platelet functions, or coagulopathy such as diffuse intravascular coagulation.

Multilineage Cytopenias

Multiple hematopoietic lineages are often involved in the same conditions that induce single-lineage low counts and have a similar etiology: immune mediate destruction, chemotherapy, metabolic deficiency such as B12 and folate, or primary bone marrow disorders such as leukemia or MDS. Bone marrow failure represents profound absence of bone marrow hematopoietic cells, leading to severe deficiencies of all blood lineages. Some inherited conditions such as Fanconi anemia manifest themselves as bone marrow failure states. These conditions usually present in childhood, but they should be part of the initial work-up for bone marrow failures in adults as well. Acquired bone marrow failure states may be secondary to chemotherapy, radiotherapy, or viral infections. Aplastic anemia is a special type of acquired bone marrow failure that results from the autoimmune-mediated destruction of HPCs. As with other autoimmune disorders, aplastic anemia usually responds to immunosuppressive therapy such as cyclosporine and antithymocyte globulin, but generally the responses are transient. Sometimes, patients with aplastic anemia have spontaneous improvement in their counts due to the emergence of a hematopoietic clone that evades the autoimmune attack [41]. The cells of this new hematopoietic clone often lack glycophosphatidylinositol (GPI)-anchored proteins. Since important complement inhibitory proteins are GPI- anchored, these new hematopoietic cells are sensitive to complement mediated lysis causing paroxysmal nocturnal hemoglobinuria. MDS can also arise in the setting of aplastic anemia. BMT remains the only curative therapy for most bone marrow failure states.

Increased Production of Hematopoietic Cells

Increased production of various blood cells generally results from clonal genetic mutations. If the mutations are associated with otherwise relatively normal differentiation, the resulting MPNs are generally accompanied by increased numbers of all blood lineages. When the mutation is also associated with a block in terminal differentiation, acute leukemia ensues with subsequent inhibition of normal hematopoiesis. Several nonmalignant conditions lead to increased production of blood cells. Stress, especially related to infections, sometimes

leads to marked granulocytosis even above 100,000 cells per μL, a condition known as a leukemoid reaction. Conditions that will elevate EPO production, such as living at high altitudes or cigarette smoking, can cause erythrocytosis. Stress and iron deficiency can elevate platelet counts.

Clonal Hematopoiesis of Undetermined Potential (CHIP)

Age-related clonal hematopoiesis has been recently described [42]. CHIP refers to otherwise normal hematopoiesis exhibiting common MDS-related genetic mutations. Although the full clinical implications of CHIP remain unclear, this condition is likely in many ways similar to the better studied and understood conditions of monoclonal gammopathy of unknown significance (MGUS) and monoclonal B-cell lymphocytosis (MBL). It is estimated that about 2–5% of patients over the age of 60 have CHIP, and like MGUS and MBL, the incidence of CHIP increases with age. Also like MGUS and MBL, CHIP appears to predispose to MDS and even acute leukemia but at relatively low rates [43, 44]. Some preclinical models of CHIP have shown abnormalities in macrophage functions and a potential increased risk of atherosclerosis [45]. Better understanding CHIP is a current area of active interest.

Conclusions

Hematopoiesis has fascinated clinicians, scientist, laymen, and philosophers alike since the beginning of times. From Hippocrates to today, clinicians interrogated the blood in an effort to find more about the physiology and pathophysiology of their patients. Research into various aspects of the cell biology of hematopoiesis is currently seeing a boost in genomic era where single-cell analysis can give information about the clonal composition of blood and stem cell behavior. Nevertheless, it remains a mystery how HSCs make differentiation choices and how they balance blood production for immediate needs and continue to do so for an individual's entire lifetime. Recent studies into the role of bone marrow microenvironment in regulation of HSC behavior and blood cell development are likely to significantly improve our understanding of both normal and abnormal hematopoiesis.

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Red Blood Cell Biochemistry and Physiology

3

Eduard J. van Beers and Richard van Wijk

Understanding the factors that regulate RBC production and development and the genetic and biochemical basis of RBC physiology is critical for an informed approach to the diagnosis and treatment of (hemolytic) anemia.

Red Blood Cell Development

(A) *Early Development*. Red blood cells (RBCs) are normally produced in the *bone marrow*. The process of RBCs development is called *erythropoiesis*. Every second 2.4 million RBCs are produced. All these RBCs are derived from *pluripotent hematopoietic stem cells* (HSC) and share a common precursor (or *progenitor cell*) with other *myeloid lineage cells* including megakaryocytes, granulocytes, monocytes/macrophages, eosinophils, and basophils. Thus, inherited or acquired abnormalities in HSCs or myeloid progenitor cells may be associated with functional or quantitative defects in multiple types of blood cells [2]. The understanding of the different stages in erythropoiesis is still evolving, and it has become clear that fetal and adult erythropoiesis are not the same.

(B) *Regulation of growth*. The growth and maturation of RBCs from the HSCs and myeloid progenitor cells are regulated by complex interplay between external signals generated by remote and/or neighboring cells and the availability of iron, folic acid, vitamin B12, and other essential organic compounds.

1. *Hematopoietic growth factors* are an important class of external signals used to regulate hematopoiesis. Multiple subtypes have been identified and characterized.
2. *Erythropoietin* (EPO) is the most important growth factor regulating erythropoiesis.
   (a) EPO is produced in the *kidney* by peritubular cells that sense tissue oxygen content. When oxygen delivery to the kidney fails (due to anemia, hypoxemia, impaired blood flow, or other causes), these renal peritubular cells rapidly increase synthesis and release of EPO.
      1. The normal rise in EPO associated with anemia may be blunted or absent in patients with renal disease.
      2. As a result, renal disease is frequently associated with anemia and is a common indication for treatment with recombinant EPO.
   (b) In response to EPO, erythroid precursors in the bone marrow are stimulated to divide and mature, resulting in increased production and release of RBC from the bone marrow.
3. Iron is essential for erythropoiesis because it is required for hemoglobin synthesis. Low levels of circulating iron, however, not only regulate hemoglobin synthesis but also suppress erythropoiesis directly by the transferrin receptor 2 which is expressed in different erythroid progenitors [7]. Conversely, the erythroid precursors produce the hormone erythroferrone which (by its suppressive effect on hepcidin production in the liver) increases iron recycling and uptake from the diet. The production of erythroferrone is increased during increased erythropoiesis, when there is an increased demand for iron [4]. However, during increased erythropoiesis with low demand for exogenous iron such as in hemolysis and ineffective erythropoiesis (see Chap. 4), erythroferrone is still produced in high quantities giving rise to pathologic high uptake of iron and subsequent secondary hemochromatosis.

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(C) *Stages of development*

1. The development stages of red blood cells are presented in Chap. 2, showing that as the erythron matures, the nucleus is extruded permitting greater deformability.
2. At the time of release from the bone marrow, the erythrocyte has not assumed the biconcave disc shape of the mature RBC. This young erythrocyte is anucleate and larger than a mature RBC and has a spherical shape characterized by the absence of central pallor.
   (a) On a Wright-stained peripheral blood smear, these cells have a faint bluish coloration in the cytoplasm (*polychromasia*) that reflects staining of residual messenger RNA directing the synthesis of hemoglobin. These cells may also contain punctate blue staining, referred to as *basophilic stippling*, which represents staining of precipitated ribosomes. Basophilic stippling is usually seen when there is abnormal heme or globin synthesis. As such it is a nonspecific sign of pathologic conditions such as hemoglobinopathies and myelodysplastic syndromes, leading to poisoning and rheumatologic diseases (see Chaps. 4, 7, and 26).
   (b) When stained with a supravital dye such as brilliant cresyl blue, the RNA and polyribosomes in these cells aggregate. These cells are identified as reticulocytes (Fig. 3.1).
3. Reticulocytes develop into fully mature RBC (smaller non-polychromatic cells with central pallor) within 1 or 2 days following release into the circulation from the bone marrow. Thus, reticulocytes are the youngest erythrocytes normally identified in the peripheral blood. An elevation in the number of reticulocytes (reticulocytosis) present in the circulation is an indication that RBC production is increased, usually in response to the loss of RBC from bleeding or *hemolysis* (i.e., shortened RBC survival).
4. From the above it is clear that in the later stages of erythropoiesis, the size and hence the mean cellular volume (MCV) of the RBC gradually reduce (see Chap. 4). A high MCV in the peripheral blood count therefore either reflects reticulocytosis, clumping of RBCs as in cold agglutinin disease and rouleaux, developmental problems such as seen in vitamin B12 deficiency, myelodysplastic syndrome, or hereditary forms of dysplastic anemias or membrane abnormalities as in hypothyroidism (see Chap. 4).

Hemoglobin: Structure and Function

(A) *Structure*

1. *Hemoglobin* (Hb) is the major protein contained in mature RBCs. A hemoglobin molecule is composed of four *globin* chains. Each globin chain is bound to a *heme* moiety containing *iron*. Two of the globin

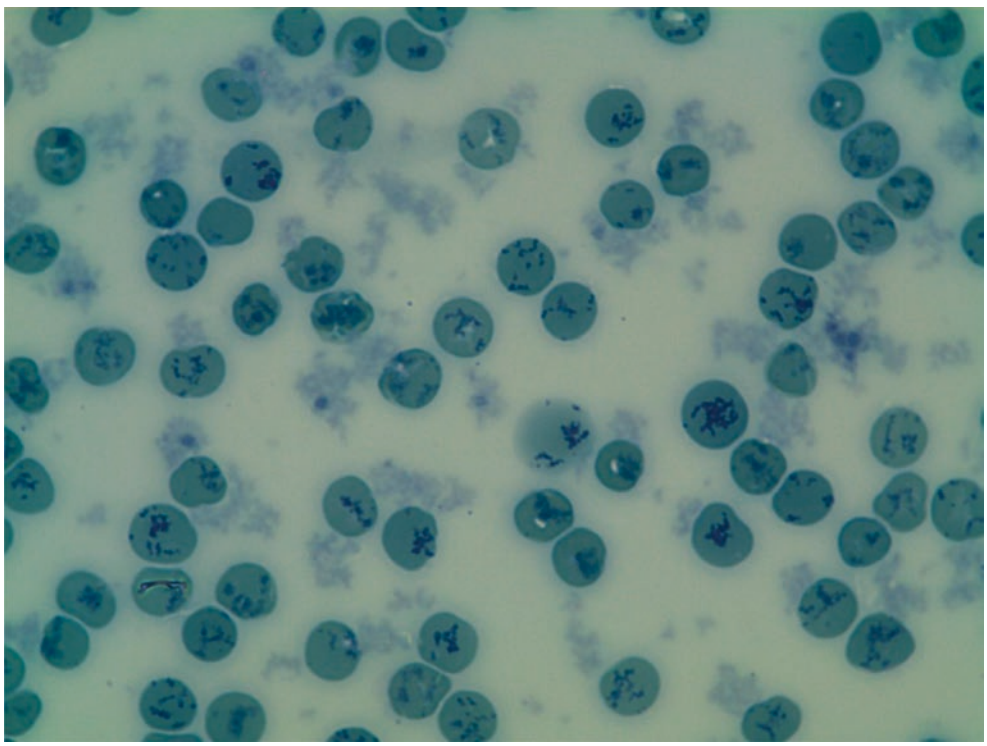

Fig. 3.1 Blue-stained reticulum of reticulocytes as identified by supravital staining (brilliant cresyl blue) in a patient with brisk reticulocytosis

chains are derived from the *alpha-globin* (α-globin) locus on chromosome 16, and the remaining two globin chains are derived from the *beta-globin* (β-globin) locus on chromosome 11.

2. Different globin chains are expressed during embryonic, fetal, and postnatal/adult stages of development. Hemoglobin molecules containing different globin chains can be distinguished from one another by electrophoresis, liquid chromatography, mass spectroscopy, and quantitative polymerase chain reaction (qPCR).
3. Hemoglobin forms
   (a) *Hemoglobin A1* is composed of two α-globin chains and two β-globin chains ($\alpha_2\beta_2$) and normally represents greater than 95% of the hemoglobin present in adult RBCs.
   (b) *Hemoglobin A2* (α2δ2) is composed of two α-globin chains and two δ-globin chains and normally represents less than 4% of the hemoglobin present in adult RBCs.
   (c) Fetal hemoglobin (Hb F) (α2γ2) contains two α-globin chains and two gamma-globin (γ-globin) chains. Hb F is the major hemoglobin present during the later stages of fetal development. Hb F has a higher oxygen affinity compared to adult HbA1. This allows oxygen transfer from the mother to the fetus in the placenta. Around the time of birth, this is not needed anymore, and expression of γ-globin and thus Hb F is suppressed. Normally Hb F levels are below 1% in adult RBCs. Higher levels of Hb F after birth are abnormal and can be a sign of stress or ineffective erythropoiesis or mutations in genes regulating Hb F suppression as well as defects in globin chain production as seen in hemoglobinopathies (see Chap. 7).
4. Genetic mutations in the α-globin or β-globin locus may result in the expression of an abnormal hemoglobin (*hemoglobinopathy*) with a different amino acid composition and aberrant migration pattern on electrophoresis. The variant hemoglobin may be functionally normal or may have physical and/or physiologic properties that differ from a normal hemoglobin molecule (see Chap. 7).
5. A second category of genetic mutations in the globin loci is characterized by a quantitative reduction in the synthesis of α-globin or β-globin chains and a net reduction in the formation of hemoglobin (*thalassemia*). Quantitative reductions of β-globin can be recognized by a compensatory increase of δ-globin (HbA2) and/or γ-globin (Hb F) on electrophoresis. As α-globin is expressed as well in HbA1 as in HbA2 (and Hb F), a quantitative reduction of its synthesis does not result in compensated production and therefore cannot be identified by electrophoresis.

(B) *Function*

1. The major physiologic role of hemoglobin is the transport of oxygen from the lungs to the tissues and subsequent export of carbon dioxide from tissues to the lungs. Oxygen binds to hemoglobin with high affinity in the oxygen-rich environment of the alveolar capillary bed and dissociates from hemoglobin in the relatively oxygen-poor environment of the tissue capillary bed. The loading and unloading of oxygen from hemoglobin are facilitated by conformational changes in the hemoglobin molecule that alter its affinity for oxygen (*cooperativity*).
2. Hemoglobin oxygenation is classically depicted by an *oxyhemoglobin dissociation* curve, where the oxygen saturation of hemoglobin is measured as a function of the partial pressure of oxygen (Fig. 3.2). A convenient measure of the oxygen affinity of hemoglobin is the partial pressure of oxygen where hemoglobin is 50% saturated (P_{50}). The P_{50} of hemoglobin varies as a function of temperature, pH, and the intracellular concentration of 2,3-diphosphoglycerate (2,3-DPG) (see also paragraph "*metabolic pathways in red blood cells: Rapoport-Luebering pathway*" below).
   (a) Acidosis (decreased pH) and elevations in RBC 2,3-DPG content stabilize the deoxyhemoglobin conformation, resulting in decreased affinity for oxygen, an increase in the P_{50}, and a right shift in the oxyhemoglobin dissociation curve. This improves oxygen delivery to peripheral tissues.
   (b) Physiologic changes in the oxyhemoglobin dissociation curve occur as adaptive responses to anemia and/or hypoxia. Intraerythrocyte 2,3-DPG levels are increased in individuals with chronic hypoxia or anemia and in individuals living at high altitude. The increase in 2,3-DPG levels results in a right shift of the oxyhemoglobin

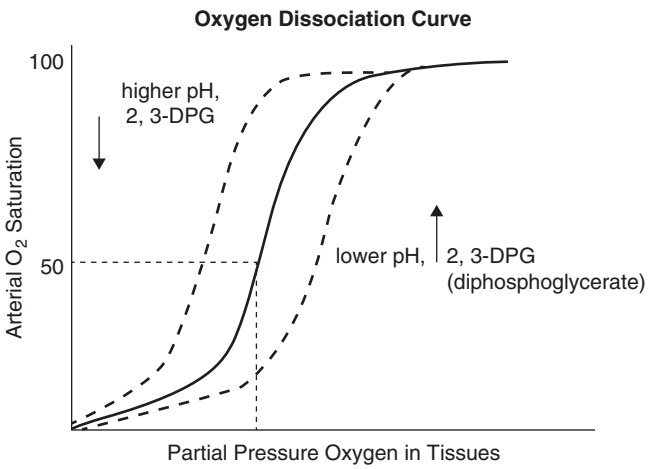

Fig. 3.2 Red blood cell oxyhemoglobin dissociation curve

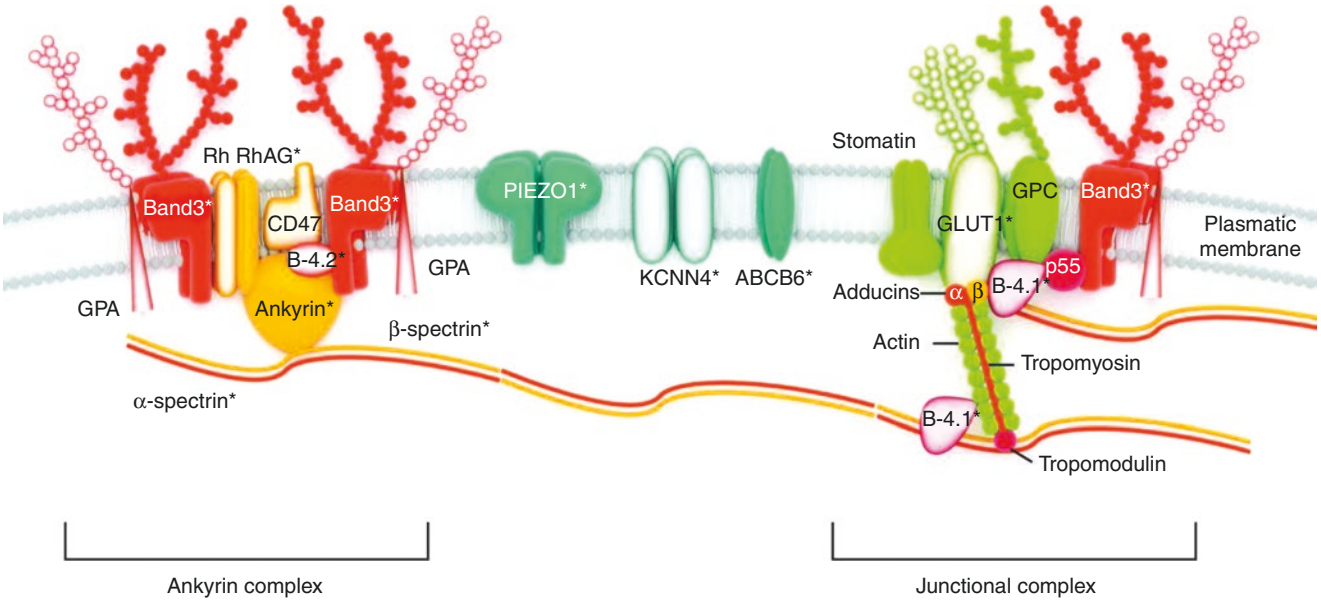

Fig. 3.3 Schematic representation of the red blood cell membrane. (From Andolfo et al. [1]) * denotes proteins in which mutations have been identified (Used with permission)

dissociation curve and the release of a greater proportion of hemoglobin-bound oxygen in tissue capillary beds. In contrast, blood that is stored outside the body for several days has greatly reduced levels of 2,3-DPG and subsequently has reduced oxygen delivery capacity (see Chapter "Blood Banking").

Red Blood Cell Membrane

(A) A mature red blood cell assumes the shape of a *biconcave disc of approximately 6 um diameter*. When viewed from above on a peripheral blood smear, it displays an area of central pallor that corresponds to the region where the upper and lower membrane surfaces of the RBC are in close proximity. The unique morphology of the red blood cell is adapted for transit through narrow capillary beds and splenic sinusoids.

1. Young, healthy red cells are highly deformable yet rapidly return to their native shape after exiting a capillary bed.
2. Red cells become more dense and thereby more rigid and less deformable as they age. This contributes to their senescence and elimination from the circulation, mainly by the spleen. The average life span of a red blood cell is 120 days.

(B) The red blood cell membrane contains numerous membrane and cytoskeletal proteins. Many of the integral (trans)membrane proteins incorporated in the lipid bilayer are located in multiprotein complexes. They are linked to the cytoskeleton, which mainly consists of spectrin tetramers composed of alpha- and beta-spectrin subunits. Spectrin tetramers form a two-dimensional network that is tethered to the phospholipid membrane through a number of structural and transmembrane proteins, of which the most important ones are ankyrin, band 3, and protein 4.2. Together they form a flexible network that enables the RBC to deform and pass even through the smallest capillaries of the human body (Fig. 3.3).

Hereditary deficiencies in function and/or quantities of membrane and cytoskeletal proteins lead to distinct morphological abnormalities such as elliptocytosis, spherocytosis, and pyropoikilocytosis. In addition, the accompanying altered properties (i.e., deformability) lead to decreased cellular survival and, hence, a variable degree of hemolytic anemia (see Chap. 4) [1].

(C) Many of the transmembrane proteins also have a transport function, thereby regulating gas exchange, ionic content, RBC membrane permeability, and RBC volume (Fig. 3.3). Pump activity is, in part, dependent on energy in the form of *adenosine triphosphate* (ATP), which is generated by *glycolysis* within the red cell (see below).

Congenital dysfunction or absence of transport proteins leads to cellular abnormalities, generally characterized by "mouth-shaped" RBCs (stomatocytes). Hereditary stomatocytosis is a highly heterogeneous group of diseases that may or may not be accompanied by hemolytic anemia (see Chap. 4).

Metabolic Pathways in Red Blood Cells

RBC metabolism is relatively simple (Fig. 3.4).

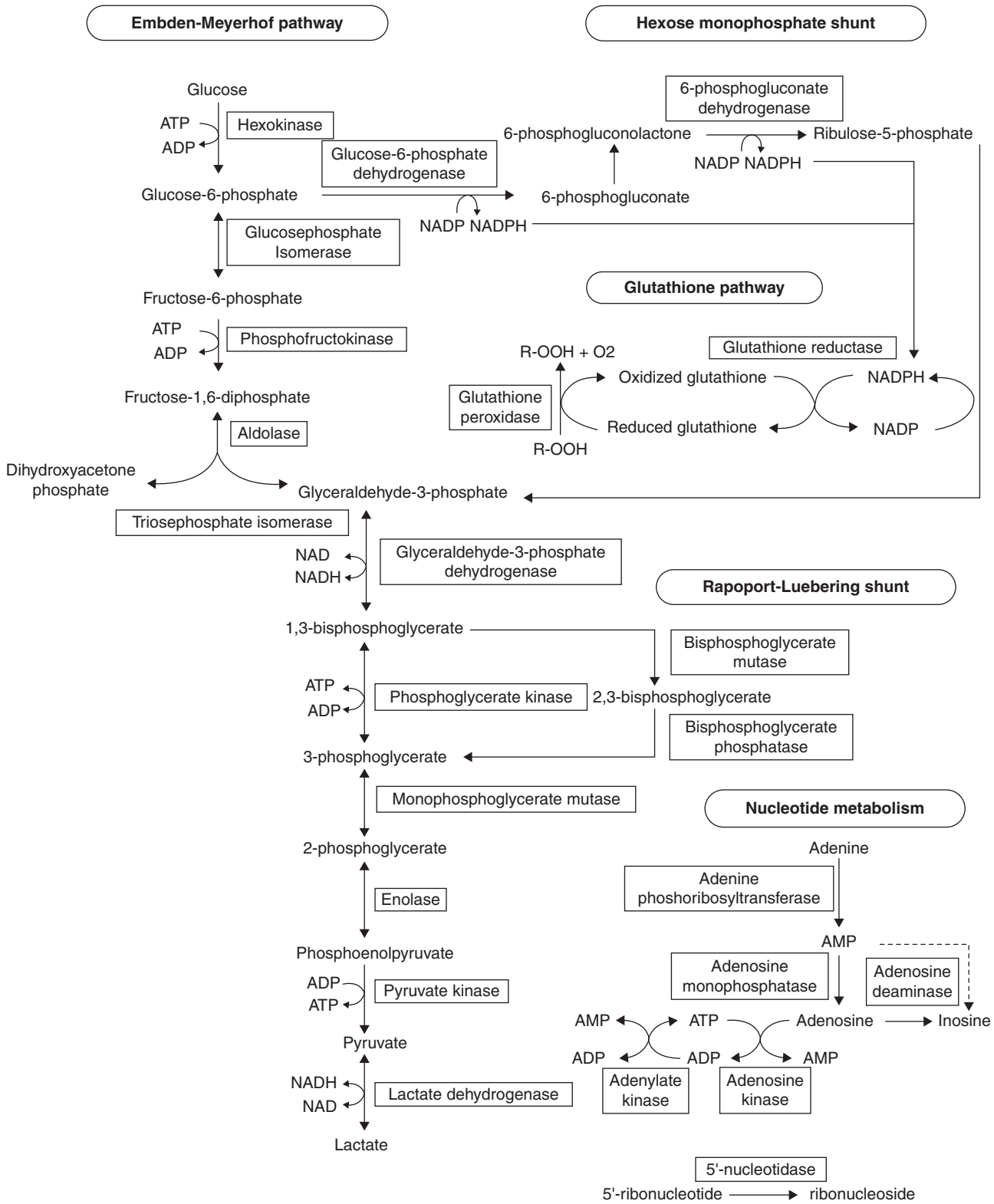

Fig. 3.4 Red blood cell glucose metabolic pathways. ADP adenosine phosphate, ATP adenosine triphosphate, G6PD glucose-6-phosphate dehydrogenase, GSH glutathione, GSSG oxidized glutathione, $HbFe^{2+}$ ferrous hemoglobin, $HbFe^{3+}$ ferric hemoglobin, NAD nicotinamide adenine dinucleotide, NADH reduced nicotinamide adenine dinucleotide, NADP nicotinamide adenine dinucleotide phosphate, NADPH (reduced) nicotinamide adenine dinucleotide phosphate. (Reprinted with permission Koralkova et al. [6])

(A) *Embden-Meyerhof pathway (anaerobic glycolysis)*. The anaerobic glycolytic pathway is the major source of ATP production in the RBC. It generates the ATP necessary to power the ionic pumps that regulate cellular ion content, hydration status, and cellular shape. Hereditary defects of glycolytic enzymes are associated with a highly variable degree of chronic hemolysis [5]. In addition, because many of these enzymes are ubiquitously expressed, hemolysis can be accompanied by a wide range of non-hematological disorders such as myopathy, neuromuscular disease, or mental retardation.

1. The hemolysis associated with defects in glycolysis is referred to as *hereditary non-spherocytic hemolytic anemia* because RBC morphology is minimally altered. *It is a rare cause of hemolytic anemia, which should be assumed after other causes, i.e., autoimmune hemolytic anemia, hemoglobin abnormalities, and membrane disorders have been ruled out*.

   The common biochemical consequence of glycolytic enzyme deficiencies is *ATP deficiency* leading to abnormalities in Na^+, K^+, Ca^{2+}, and volume regulation. *In addition, accumulation of glycolytic intermediates occurs which may affect other steps in the glycolytic pathway*. The precise mode of destruction of the RBCs is unknown, but typically affected RBCs are susceptible to retention and destruction in the spleen and to a lesser extent in the liver. The most common enzyme defect of glycolysis is pyruvate kinase deficiency [3].
2. In a bypass of glycolysis (the *Rapoport-Luebering pathway*), 2,3-diphosphoglycerate (2,3-DPG) is produced. 2,3-DPG is an important regulator of the oxygen affinity of hemoglobin. Defective function of this pathway and the consequent reductions in 2,3-DPG levels cause a left shift in the oxyhemoglobin dissociation curve. This shift impairs oxygen delivery to the tissues and increased EPO production and red cell mass (congenital secondary erythrocytosis). *Conversely, the reduced oxygen affinity caused by* increased levels of 2,3-DPG as seen in pyruvate kinase deficiency is associated with increased tolerance of low hemoglobin levels.

(B) *Hexose monophosphate shunt*. A small portion of glucose is catabolized through the hexose monophosphate shunt. The RBC is designed to transport high concentrations of oxygen, an extremely reactive molecule with the potential to cause oxidative damage to RBC proteins, including hemoglobin. Therefore, the RBC is equipped with highly efficient defense mechanisms to protect itself from *oxidative stress*. *Reduced glutathione* is present in high amounts and a critical molecule in the detoxification of *hydrogen peroxide*, the primary chemical intermediate involved in oxidative damage. Glutathione is maintained in its reduced state by NADPH generated by the hexose monophosphate shunt. The most common enzyme defect of the pentose phosphate shunt is glucose-6-phosphate dehydrogenase (G6PD) deficiency. RBCs of patients with G6PD deficiency hemolyze when exposed to oxidants, e.g., from drugs or food (broad beans) which cause favism. It is the most common enzymopathy in man effecting 400 million individuals worldwide. Many lists of oxidizing medications can be found online, but the number of drugs that cause clinical relevant hemolysis is limited (see Luzatto and Seneca [6]).

(C) *Methemoglobin reduction pathway*. The methemoglobin reduction pathway serves to maintain the iron of hemoglobin in its ferrous (Fe2+) state. Defects in this pathway lead to methemoglobinemia. Patients with methemoglobinemia may present with typical cyanotic discoloration of skin with or without shortness of breath. Worsening of clinical symptoms may be caused by certain drugs, such as local anesthetics, oxidative drugs, and nitrates.

Summary

Red blood cells are derived from precursor cells in the bone marrow. The major physiologic function of RBC is oxygen transport. Oxygen status has important direct effects on RBC production by the bone marrow and the biochemical properties of hemoglobin. The metabolic pathways of the RBC serve to provide a simple, reliable source of ATP from glycolysis and maintain a steady level of reducing power to protect RBC components from oxidative damage.

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Anemia: Clinical Approach

4

Peter W. Marks

Definition of Anemia

The oxygen carrying capacity of red blood cells is provided by hemoglobin. Anemia is present when this value in blood falls below age- and gender-appropriate normal values, which are defined by values two standard deviations below the mean for normal individuals of similar age and gender (i.e., outside the 95% confidence interval for the population). The volume of red blood cells reported as a percentage of the total volume of blood present is the hematocrit. This value is commonly used as an alternative method for defining anemia. In most cases the two values relate to one another roughly by a factor of three (hemoglobin × 3 ≈ hematocrit).

The hemoglobin value in children is lower than that of adults [1]. During puberty, an increase in hemoglobin occurs in males due to androgenic steroids. The normal range for hemoglobin in males is therefore higher than for females (Table 4.1). In maturity, the difference between men and women decreases. In particular, over the two decades after 70 years of age, men's hemoglobin levels drop by about 1 g/dL [2]. Thus, the mean hemoglobin concentration for a 90-year-old man is about 14.1 g/dL compared to about 13.8 g/dL for a 90-year-old woman.

Major categories of anemia are:

- Acute blood loss
- Inadequate production of red blood cells
- Destruction of red blood cells

Acute Blood Loss

Loss of blood acutely may not be associated with an immediate decline in hemoglobin concentration, since this loss consists of an equivalent proportion of cellular elements and plasma. However, after volume repletion a decrease in the hemoglobin concentration or hematocrit proportional to the amount of blood lost may be observed.

Inadequate Production

There are a number of entities commonly associated with an inadequate production of red blood cells. Some of these affect other cell lineages as well.

- *Iron deficiency anemia* is the most common cause of anemia globally (see Chap. 5) [3]. With rare exception, iron deficiency anemia in adults results from chronic blood loss. In women, menstrual blood loss may explain its development (and it is present in about 5% of menstruating females in the United States). In men, the identification of iron deficiency anemia should always provoke a search for blood loss. Even in younger women, consideration of gastrointestinal blood loss may merit consideration, depending on individual circumstance [4].
- *Anemia of inflammation (also known as the anemia of chronic disease)* is commonly encountered in association with a variety of conditions, including serious infections, rheumatologic disease, diabetes mellitus, and malignancy. In this condition the iron regulatory protein hepcidin decreases the ability of the reticuloendothelial system to release stored iron [5]. The lack of bioavailable iron essentially mimics the situation with iron deficiency anemia from blood loss. When combined with the suppressive effect of certain cytokines on red blood cell production, this circumstance leads to a mild to moderate anemia that may share some morphologic features with iron deficiency.
- *Anemia of renal disease* results from erythropoietin deficiency. The synthesis of this hormone is regulated by the oxygen tension in the periglomerular cells of the kidney. Hypoxia drives the synthesis of erythropoietin and its

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Table 4.1 Normal range for red blood cell parameters

Parameter		Adult normal range
Red blood cell number (RBC)	Male	$4.5–5.9 \times 10^{12}$/L
	Female	$4.0–5.0 \times 10^{12}$/L
Hemoglobin (Hgb or Hb)	Male	13.5–17.5 g/dL
	Female	12–16 g/dL
Hematocrit (Hct)	Male	41–53%
	Female	36–46%
Mean corpuscular volume (MCV=Hct/RBC)		80–100 fL
Mean corpuscular hemoglobin (MCH=Hgb/RBC)		26–34 pg
Mean corpuscular hemoglobin concentration (MCHC=Hgb/Hct)		31–37 g/dL
Red cell distribution width (RDW=(SD[a] of MCV/MCV) ×100)		11–15%

[a]*SD* standard deviation

release into the bloodstream, which stimulates the maturation and development of erythrocyte precursors in the bone marrow. These activities result in an increase in red blood cell mass, bringing additional oxygen to the kidney, and ultimately completing the feedback loop by downregulating production of erythropoietin [6]. A reduction in renal function is generally accompanied by a reduction of erythropoietin production.

- *Endocrine anemias* result from deficiencies or excess of hormones that contribute to blood cell development. To provide a few examples, hypothyroidism may be associated with a mild to moderate anemia sometimes associated with macrocytosis; adrenal cortical insufficiency may be accompanied by a normocytic anemia; and decreased levels of serum testosterone may lead to a mild anemia in males [7].
- *Pure red cell aplasia* in children may be the result of heritable disorders, such as congenital hypoplastic anemia (Diamond-Blackfan anemia), or may be the apparent result of infection with a virus (e.g., Parvovirus B19) or an immunologic phenomenon (e.g., as seen in systemic lupus erythematosus) [8]. In contrast to aplastic anemia, in which two or more cell lineages are affected, pure red cell aplasia is characterized by preservation of the white blood cell count and platelet count.
- *Bone marrow replacement* is also known by the term myelophthisis. In this case, the blood forming bone marrow space is taken over by cells or material that should not be there. Causes of bone marrow replacement include hematologic malignancies such as leukemia or lymphoma, metastatic cancer (most commonly breast or prostate), infection with fungi or other microorganisms, and fibrosis such as that which may occur in conjunction with primary myelofibrosis.
- *Folate and vitamin B12 deficiency* are two types of megaloblastic anemia that lead to maturation abnormalities in all three cell lineages. These disorders share in common the pathophysiology of impaired synthesis of DNA [9]. Folate deficiency is generally related to inadequate dietary intake or to increased requirements due to red blood cell hemolysis. The situation for vitamin B12 (also called cobalamin) is more complex. Vitamin B12 is released from food in the acidic environment of the stomach and binds to the intrinsic factor that is secreted by the parietal cells in the stomach (see Chap. 6). The intrinsic factor-vitamin B12 complex then travels to the terminal ileum where it is absorbed. Vitamin B12 deficiency may result from several different causes including inadequate stomach acidity, pernicious anemia (an autoimmune phenomena destroying the parietal cells that synthesize intrinsic factor), structural lesions in the terminal ileum due to conditions such as Crohn's disease, and from surgical resection of portions of the GI tract. Inadequate dietary intake is generally only observed in vegans.
- *Sideroblastic anemia*s represent an uncommon group of hereditary and acquired disorders in which iron is not effectively used in hemoglobin synthesis leading to iron accumulation in the mitochondria of red blood cell precursors [10]. The deposition of iron in mitochondria leads to the morphologic entity of ringed sideroblasts in the bone marrow when it is stained for iron. Exactly as the name implies, ringed sideroblasts are cells in which iron-laden mitochondria encircle at least one-third of the circumference of the erythroblast nucleus. Usually at least five iron-laden mitochondria need to be seen encircling the nucleus to make diagnostic criteria. Hereditary forms of sideroblastic anemia are rare and may be X-linked, autosomal dominant, or recessive. Acquired forms may occur after exposure to drugs (e.g., cyclosporine, vincristine) or toxins (ethanol).

Destruction

Normally red blood cells circulate for about 100 to 120 days before they are cleared by the reticuloendothelial system. Premature red blood cell destruction may result from intrinsic defects such as abnormal hemoglobin molecules, cytoskeletal proteins, or enzymes. It may also result from defects extrinsic to the erythrocyte, including mechanical forces and antibody or complement-mediated red cell breakdown.

- *Hemoglobinopathies* include alpha- and beta-thalassemia, disorders in which there are insufficient production of one of the globin chains, and the structural mutations (See Chap. 7) [11, 12]. Alpha- or beta-thalassemia traits (loss of two alpha genes, or one beta gene) are common causes of microcytosis associated with little or no anemia.

Microcytosis occurs in thalassemia because the deficiency in hemoglobin stimulates additional cell divisions of erythrocyte precursors in order to try to preserve the hemoglobin concentration. Thalassemia trait is particularly common in individuals from Africa, Asia, and the Mediterranean Basin. Among most commonly encountered structural mutations is a glutamine to valine substitution at position 6 of the beta globin gene. This change results in the production of hemoglobin S, which tends to polymerize in its deoxygenated state [13]. Heterozygotes with one copy of hemoglobin S (sickle cell trait) are relatively protected against infection with the malaria parasite. This structural mutation thus provides a survival advantage, and selective pressure has led to persistence of the mutation. Homozygotes with two copies of hemoglobin S have sickle cell anemia, a serious life-defining hematologic disorder. Many other structural mutations exist and can result in changes in the properties of hemoglobin, such as reducing its solubility (e.g., hemoglobin C and hemoglobin D), decreasing its stability, or changing its oxygen affinity.

- *Red blood cell membrane defects* result from a variety of different defects affecting the red blood cell cytoskeleton or the membrane itself (See Chap. 3) [14]. Maintenance of the normal biconcave shape requires intact cytoskeletal architecture. Defects in any of the proteins involved, including ankyrin, spectrin, and band 3, among others, lead to changes that reduce the resiliency of red blood cells as they pass through the narrow passageways in the spleen and other portions of the circulation. This initially leads to the formation of spherocytes and ultimately resulting in hemolysis. The resulting disorder, hereditary spherocytosis, is most common in individuals of Northern European descent. Liver disease is the most common cause of an acquired red cell membrane defect and results in abnormal cells noted on examination of the blood smear (codocytes or target cells). Abnormalities in the lipid composition of the red blood cell membrane result in cells that are abnormally stiff and unable to rebound from deformities that arising from transit of the circulation. Paroxysmal nocturnal hemoglobinuria (PNH) represents a rare type of acquired membrane defect that is derived from a stem cell defect leading to the reduction or absence of phosphatidylinositol glycan-linked membrane proteins [15]. The lack of one such erythrocyte phosphatidylinositol glycan-linked membrane protein, decay accelerating factor, is associated with the hemolysis of red blood cells through the unopposed constitutive activation of components of the complement cascade (See Chap. 9).
- *Red blood cell enzyme defects* are potentially the most common red cell abnormalities globally (See Chap. 8). Glucose-6-phosphate dehydrogenase (G-6-PD) is the enzyme required for function of the hexose monophosphate shunt in the red blood cell. This pathway provides the red blood cell with reduction capacity against oxidant stress. The gene encoding G-6-PD is on the X chromosome. Mutations in G-6-PD are very common and have been preserved in populations because of their relative protection against infection with the malaria parasite, like sickle cell anemia [16]. The two most common mutations cause a reduction in cell enzyme activity in the aging erythrocyte (A-variant) or result in absent function throughout the red cell life span (Mediterranean variant). Those carrying mutations in G-6-PD (especially males, since this is an X-linked trait) have red cells that are susceptible to hemolysis under conditions of oxidant stress. Common causes of oxidant stress include medications such as antimalarials, dapsone, and sulfamethoxazole.
- *Mechanical causes of hemolysis* may result from microscopic or macroscopic forces. Microangiopathic hemolytic anemias (MAHA) include disseminated intravascular coagulation (DIC), thrombotic thrombocytopenic purpura (TTP), and hemolytic uremic syndrome (HUS) [17]. In these conditions abnormalities in the microvasculature result in shearing of the red blood cell and the formation of red cell fragments called schistocytes. Certain uncommon infections, such as those with clostridia or bartonella species, can also be associated with the production of toxins that lead to red blood cell destruction through what is essentially mechanical disruption of the membrane. Accelerated or malignant hypertension and vasculitis are additional etiologies producing mechanical destruction of red blood cells. Malfunctioning mechanical valves, perivalvular leaks, as well as long-distance running can all result in the mechanical destruction of red blood cells by mechanical trauma [18].
- *Autoimmune hemolytic anemia* results from the formation of antibodies that bind to the red blood cell and either fix complement resulting in its destruction in the circulation (intravascular hemolysis) or result in clearance in the reticuloendothelial system and spleen (extravascular hemolysis) (See Chap. 9). Warm autoantibodies are often idiopathic but may be associated with hematologic malignancies such as chronic lymphoid leukemia or rheumatologic disorders such as systemic lupus erythematosus [19]. Similarly, cold autoantibodies may be idiopathic or associated with lymphoproliferative or rheumatologic disorders [20]. Transient cold agglutinins may be associated with infectious mononucleosis and infection with mycoplasma.
- *Alloimmune hemolytic anemia* results from exposure of an individual to foreign red blood cells. In children and adults, this most commonly results from blood transfusion in which there are mismatched minor antigens.

Symptoms and Signs of Anemia

The symptoms and signs of anemia correlate with the reduction in the oxygen carrying capacity of the blood and the ability of the affected individual to compensate for this defect. Rapid loss of an even moderate amount of blood may be associated with shock and collapse of the circulatory system. Blood loss that occurs gradually over time is often reasonably well tolerated until relatively more severe, as adaptive changes can help compensate for the anemia. The corollary to this is that individuals with anemia that has developed gradually often do not require, and in fact can be harmed by aggressive intervention with the administration of the blood (e.g., pulmonary edema may develop with overaggressive transfusion).

The most common symptom of anemia that patients tend to report is fatigue. Decreased exercise tolerance and dyspnea on exertion may be noted by individuals as the extent of the anemia worsens. Certain types of anemia, such as iron deficiency anemia, may be associated with pica (e.g., ice chips, Argo starch), the dietary intake of non-food substances. When anemia is associated with a microangiopathy, bruising or bleeding may be reported due to the accompanying thrombocytopenia or coagulopathy.

Signs of anemia may include conjunctival pallor and a pale complexion. Tachycardia and/or systolic flow murmurs may be present if the degree of anemia is pronounced. Iron deficiency anemia may be associated with cracking of the edges of the lips (angular chelitis) and with spooning of the nails (koilonychia) [21]. Right upper quadrant pain may develop as a result of cholecystitis due to the formation of calcium bilirubin gallstones in the presence of hemolysis. Splenomegaly may result from chronic congenital or acquired hemolysis leading essentially to hypertrophy of the reticuloendothelial system or from extramedullary hematopoiesis (the presence of maturing hematopoietic precursors outside of the bone marrow), which may be associated with some myeloproliferative disorders [22].

Laboratory Diagnosis

A systematic approach to the diagnosis of anemia is essential in order to minimize unnecessary diagnostic testing and to arrive expediently at the correct diagnosis. Despite the availability of sophisticated diagnostic testing, careful consideration of the information provided by the different parameters included in the complete blood count (CBC), and a review of a well-prepared peripheral blood smear often provides a great wealth of diagnostic information. The CBC includes a variety of red blood cell indices. These calculated values are very important to classify anemia and guides one toward the differential diagnosis of anemic patient (Table 4.1) [23, 24].

When evaluating anemia, the two red blood cell indices that provide the greatest diagnostic information are the mean corpuscular volume (MCV) and red cell distribution width (RDW). MCV, which is calculated by the ratio of hematocrit (HCT) to red blood cell count (RBC), denotes red blood cell size in femtoliters (10^{-15} L). It may be small (microcytic), normal (normocytic), or large (macrocytic) depending on where it falls relative to the normal range. The red cell distribution width, which is actually the coefficient of variation of the mean corpuscular volume (mean erythrocyte size) [(standard deviation of MCV/MCV) ×100], may be either normal or elevated. When the MCV and RDW are used in combination, the various types of anemia tend to fall in one of the six possible categories, although overlap obviously exists (Table 4.2).

Reticulocyte Count

The reticulocyte count measures the production and release of newly formed red blood cells. It should be obtained along with the CBC and peripheral blood smear in the evaluation of anemia, as it provides complementary information [25]. Reticulocytes normally contain residual RNA for about the first day that they are present in the circulation. The reticulocyte count is obtained by supravital staining of red cells with dyes that bind to nucleic acid (e.g., new methylene blue or

Table 4.2 MCV and RDW in the categorization of anemia

	Low MCV	Normal MCV	High MCV
Normal RDW	Chronic disease	Acute blood loss	Aplastic anemia
	Thalassemia trait	Inflammation	Chronic liver disease
		Renal disease	Various medications
High RDW	Iron deficiency	Early iron deficiency	B12 deficiency
	Sickle beta-thalassemia	Early B12 deficiency	Folate deficiency
		Early folate efficiency	Immune hemolysis
		Sickle cell anemia	Chronic liver disease
		SC disease	Myelodysplasia
		Chronic liver disease	
		Myelodysplasia	

ethidium bromide) in order to identify the newly released erythrocytes. Although previously determined by manual methods, the reticulocyte count is now often determined by automated methods. Since the normal lifespan of red blood cells is 100–120 days, it follows that in the absence of anemia the normal reticulocyte count is about 1%, which corresponds to an absolute reticulocyte count of 25,000–50,000/μL.

When the reticulocyte count is reported as a percentage, it must be corrected for the degree of anemia. Since the same number of reticulocytes diluted in half the number of red blood cells will double the apparent percentage present, the following correction must be applied:

$$\text{Reticulocyte count}(\%) \times \text{Patient's hematocrit} / \text{Normal hematocrit for age} = \text{Corrected reticulocyte count}(\%) \text{ or Reticulocyte index}$$

In the presence of anemia, the absolute reticulocyte count should be at least 100,000/μL. This value corresponds to a reticulocyte index of at least 2% and represents an appropriate response to blood loss or to red blood cell destruction (hemolysis). Alternatively, a decreased reticulocyte count indicates the presence of a hypoproliferative process or a red blood cell maturation abnormality. Anemias can be classified by whether or not they are associated with an appropriate reticulocyte response (Table 4.3) or by a combination of the observed MCV and reticulocyte count (Table 4.4).

Table 4.3 The reticulocyte count and causes of anemia

Reticulocytes <100,000/μL or reticulocyte index <2%	Reticulocytes ≥100,000/μL or reticulocyte index ≥2%
Hypoproliferative anemias	Appropriate response to blood loss
Iron deficiency anemia	
Anemia of acute inflammation	Hemolytic anemias
Anemia of renal disease	Hemoglobinopathies
Endocrine anemias	Membrane defects
Pure red cell aplasia	Enzyme defects
Bone marrow replacement	Mechanical causes
	Autoimmune hemolytic anemia
Maturation defects	Alloimmune hemolytic anemia
Folate deficiency	
B12 deficiency	
Sideroblastic anemia	

Peripheral Blood Smear

The wealth of information provided by the CBC and reticulocyte count noted above is greatly complimented by review of the peripheral blood smear [26]. Sometimes this may be all that is required in order to reach a diagnosis, and often carefully looking at the peripheral blood smear significantly narrows down the diagnostic entities under consideration. This can help appropriately target further laboratory investigation.

A normal red blood cell is about the size of a lymphocyte nucleus (about 8 μM). The area of central pallor occupies about one-third of the overall diameter. Hypochromic cells have too much and hyperchromic too little central pallor. *Anisocytosis* is the term used to describe variation in cell size, *poikilocytosis* describes the variation in cell shape, and anisopoikilocytosis defines the two combined. Terminology describing the more common morphologic abnormalities of the red blood cell and their associated features are listed in Table 4.5.

Integration of Information from the CBC, Reticulocyte Count, and Peripheral Smear

Because the MCV, RDW, reticulocyte count, and peripheral smear all provide complementary information, integration of

Table 4.4 MCV and reticulocyte count scheme for classification of anemia

	MCV 80–100		
MCV <80	Low reticulocytes	High reticulocytes	MCV >100
Iron deficiency	Anemia of acute inflammation	Acute blood loss	B12 deficiency
Thalassemias	Renal disease	Hemolytic anemias	Folate deficiency
		Hemoglobinopathies	
		Enzyme defects	
		Mechanical anemias	
		Autoimmune hemolytic anemias	
Anemia of acute inflammation	Endocrine anemias	Alloimmune hemolytic anemias	Liver disease
Sideroblastic anemias	Aplastic anemia		Thyroid disease
Lead poisoning	Pure red cell aplasia		
	Bone marrow failure		
	Leukemia		
	Myelodysplastic syndromes		
	Myeloproliferative syndromes		

Table 4.5 Morphologic features of the erythrocyte

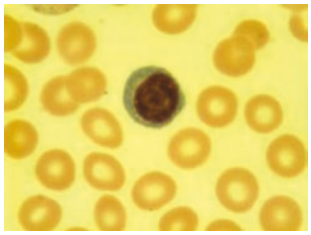
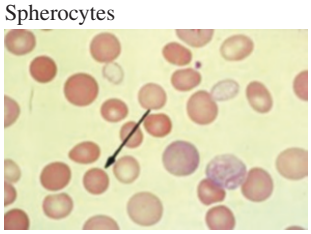
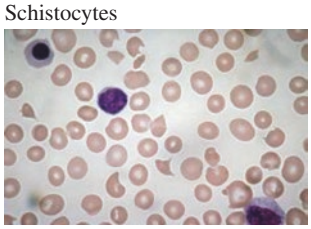
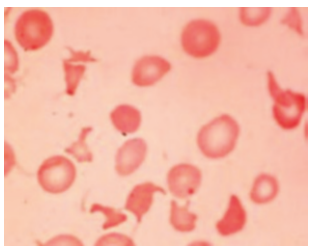
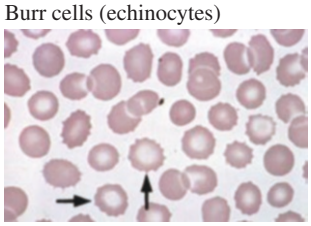
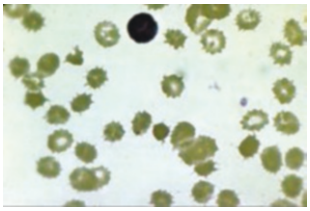
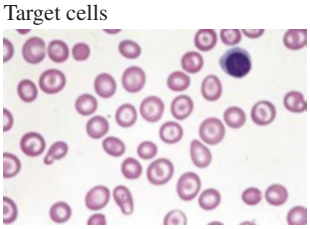
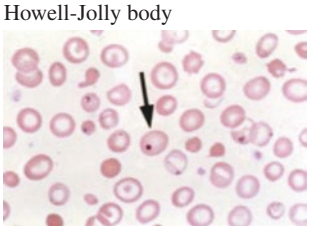
Normal erythrocyte 	Biconcave disk about 8 μM in diameter (about the size of the nucleus of a normal lymphocyte) Area of central pallor about 1/3 of the overall diameter
Spherocytes 	Loss of central pallor of the RBC DDx: immune hemolysis, hereditary spherocytosis
Schistocytes 	RBC fragmentation DDx: DIC, TTP, HUS, mechanical hemolysis
Bite cells 	Bites taken out of the RBC membrane DDx: hemolysis w/G-6-PD deficiency, unstable hemoglobins
Burr cells (echinocytes) 	Undulations of the RBC surface on blood smear DDx: uremia

Table 4.5 (continued)

Spur cells (acanthocytes) 	Spikes off of the RBC surface with loss of central pallor DDx: liver disease, abetalipoproteinemia
Target cells 	RBC that look like bull's eye targets DDx: liver disease, hemoglobin C
Howell-Jolly body 	Single purple inclusion in the RBC Represent small nuclear remnants DDx: asplenia or functional asplenia, very brisk hemolysis

these parameters leads to the correct diagnosis or provides significant insight as to the differential diagnosis (Fig. 4.1). For example, an anemia presenting with a low MCV and high RDW in which the reticulocyte count is low is almost always iron deficiency anemia. The finding on peripheral blood smear of hypochromic, microcytic cells along with wide variation in cell shape and size makes the diagnosis very likely. Iron studies and a ferritin level can then be obtained. At the other end of the spectrum, a newly occurring anemia that presents with a high MCV and high RDW in which the reticulocyte count is high is most likely to be associated with autoimmune hemolysis. A finding of spherocytes on examination of the peripheral blood smear would be highly suggestive of this diagnosis and would provide further laboratory investigation such as obtaining a direct antiglobulin test.

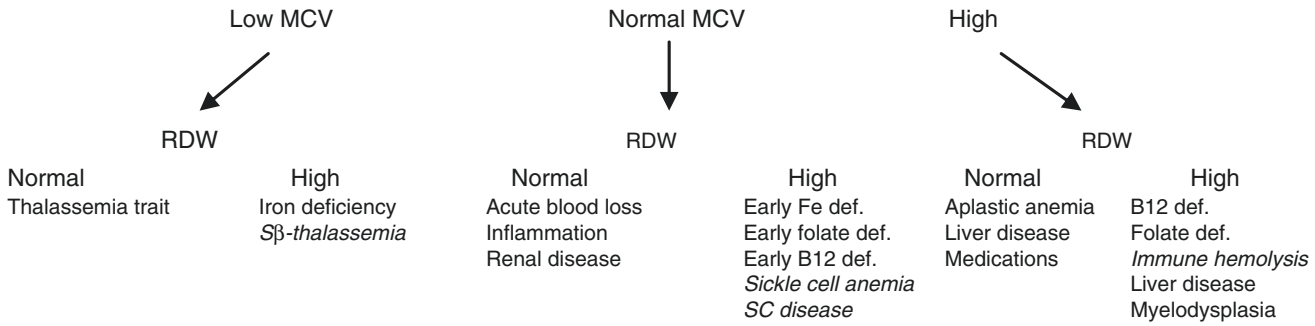


Fig. 4.1 Integration of information from the mean corpuscular volume (MCV) and red cell distribution width (RDW)

Summary

Anemia is the most commonly encountered hematologic abnormality in clinical practice. Careful consideration of the information provided by the complete blood count and reticulocyte count in conjunction with review of the peripheral blood smear often provides significant insight into the differential diagnosis and by guiding further testing expedites appropriate diagnosis with a minimum number of tests.

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Iron Deficiency

5

Gordon D. McLaren

Introduction

Iron deficiency is the most common cause of anemia worldwide. In the United States, iron deficiency is common among women of childbearing age and in infancy, when growth is rapid and an enhanced iron supply is required for an increasing red blood cell (RBC) mass. In men and non-menstruating women, iron deficiency is most often a result of blood loss, usually from the gastrointestinal (GI) tract. Anemia occurs once body iron stores have been exhausted. This chapter will focus on iron metabolism, the causes of iron deficiency and iron-deficiency anemia, its pathophysiology and clinical features, approach to diagnosis and differentiation from other conditions, and management.

Iron Metabolism

(A) *Body iron distribution*

i. Normal total body iron in adult males is approximately 4 g (~50 mg/kg body weight); in menstruating females, body iron is lower, about 2.5–3 g (~35 mg/kg).
ii. Iron is essential for cellular function, and iron-containing compounds are found in all cells. Most of the body's iron is contained within the heme moiety of hemoglobin, with smaller amounts in myoglobin and other heme proteins and cellular enzymes. Storage iron is sequestered in a nontoxic form in ferritin and hemosiderin within the reticuloendothelial system (RES) and liver. A small but essential amount of iron circulates in the plasma bound to transferrin.

1. The majority of body iron is found in the form of heme proteins.
   (a) Since 1 mL of RBCs contains ~1 mg of iron, and the average adult male has a red cell mass of about 2.5 L, the amount of iron contained in hemoglobin in the circulation is about 2.5 g. Women have a smaller amount, approximately 1.5–2.0 g, in this form.
   (b) Myoglobin in muscle comprises 3% (about 0.1 g) of total body iron.
   (c) Some other heme proteins, such as cytochromes, catalases, and peroxidase, make up <1% of total body iron.
2. ~0.1% (3 mg) of body iron circulates in the plasma bound to transferrin, the major iron transport protein.
3. The remainder of body iron is in storage (about 1 g in men and 300 mg in women) in the form of ferritin and hemosiderin, primarily in the liver and RES. Iron can be mobilized from storage when there is a need for increased supplies to restore hemoglobin levels in the event of blood loss.
   (a) *Ferritin* is the major form of storage iron, which can be mobilized for increased demands. It consists of an outer protein shell comprised of 24 subunits surrounding an iron core containing up to about 4500 iron atoms per ferritin molecule.
   (b) *Hemosiderin* is composed of aggregates of ferritin molecules that have partially lost their protein shells. It is a more stable and less soluble form of storage iron. However, all the iron stored in both ferritin and hemosiderin can be mobilized if needed to replace losses.

The original version of the chapter was revised: This chapter was inadvertently published with few errors. The correction to the chapter is available at https://doi.org/10.1007/978-3-319-97873-4_39

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(B) *Overview of body iron exchange*

i. Most body iron is taken up by RBC precursors in the bone marrow and incorporated into hemoglobin. Hemoglobin iron is removed from senescent RBCs taken up at the end of their lifespan by macrophages. The iron is removed from the heme ring and recycled to the bone marrow for hemoglobin production. Thus, iron is continuously recycled between the RES and the bone marrow, and this recycling system supplies most of the iron needed for RBC production.
   1. The majority of circulating iron derives from destruction of approximately 20 mL of red cells daily in the RES, which liberates about 20 mg of iron (Fig. 5.1).
   2. Iron from senescent RBCs is transported from the RES via the plasma, bound to transferrin. Release of iron from macrophages is mediated by ferroportin (Fpn, Fig. 5.1), the only known cellular iron exporter. In turn, the abundance of ferroportin on the macrophage plasma membrane is regulated by hepcidin, a liver-derived polypeptide hormone that binds ferroportin, leading to its internalization and degradation [1]. Hepcidin levels are reduced in iron deficiency and increased in certain other conditions such as iron overload and inflammation. (The roles of hepcidin and ferroportin will be discussed further in Sect. C.(i)2., below, and in section "The Anemia of Inflammation (Anemia of Chronic Disease)" below.)
   3. A further 1–2 mg of iron per day is derived from dietary iron absorption and is also transported via the plasma. The iron taken up from dietary sources replaces small amounts of iron lost daily from the body, mainly by exfoliation of epithelial cells (see Sect. C. (iii), below).
   4. Circulating iron is rapidly removed from the plasma, primarily by nascent RBC precursors in the bone marrow, with smaller portions going to other dividing cells and to iron stores.

(C) *The iron cycle*

i. *Intestinal iron absorption*
   1. The average iron content of the Western diet is 10–20 mg/day, of which only about 10% is absorbed.
      (a) Heme iron is absorbed more efficiently than nonheme iron and is the best source of dietary iron.
         - Heme is absorbed intact from the gut, mainly in the duodenum, and the iron is removed from the heme ring within the enterocytes and enters the same pool as nonheme iron.
         - Heme iron accounts for 10–15% of the iron in a non-vegetarian Western diet.
      (b) Nonheme iron is less well absorbed.
         - Ferric (Fe^{3+}) iron in food must be reduced to ferrous (Fe^{2+}) iron before absorption. The low-pH environment in the stomach solubilizes the iron and helps maintain it in the ferrous state during transport to the proximal duodenum.
         - Reduction of ferric to ferrous iron is also promoted by the mucosal ferrireductase enzyme, duodenal cytochrome b (DCYTB).
         - Nonheme iron absorption is enhanced by formation of complexes with peptides from meat.
         - Vitamin C enhances absorption of nonheme iron by chelating ferrous iron at acid pH in the stomach and maintaining its solubility in the alkaline pH of the duodenum, where iron absorption takes place.
         - Nonheme iron can be bound to food phytates in vegetable fiber and polyphenols in tea, which impair absorption.
   2. Iron absorption is influenced by iron stores, and absorption is enhanced in patients with iron defi-

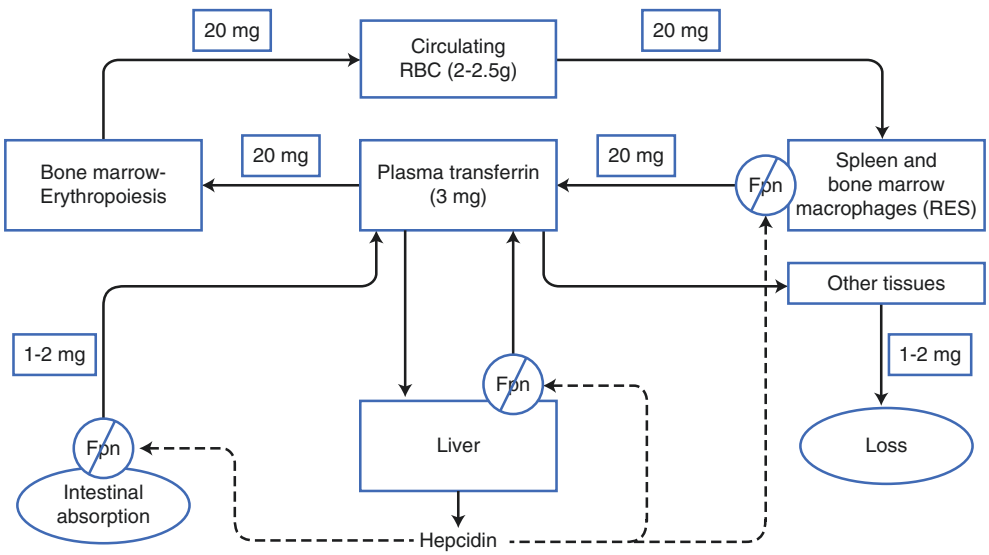

Fig. 5.1 Iron kinetics: daily iron exchange and regulation by hepcidin. See text for description. Fpn: ferroportin

ciency. This modulation is mediated by the circulating hormone hepcidin, the central regulator of iron metabolism (Fig. 5.1).

(a) Hepcidin binds to ferroportin, the only known cellular iron exporter, on the basolateral membrane of duodenal enterocytes, causing it to be internalized and degraded.
(b) Ferroportin is more abundant when hepcidin levels are lower, leading to increased transfer of iron to the systemic circulation.
(c) Hepcidin production is increased in the presence of excess iron stores or inflammation (section "The Anemia of Inflammation (Anemia of Chronic Disease)") and decreased in iron deficiency or hypoxia.
(d) Increased erythropoietic activity, as occurs in hemolytic conditions, increases iron absorption especially when accompanied by increased ineffective erythropoiesis (e.g., in thalassemia major or intermedia).

ii. *Cellular iron uptake and distribution*
1. Transferrin-bound iron is delivered primarily to red cell precursors in the bone marrow via binding of transferrin (Tf) to specific transferrin receptors (TfRs) on the outer cell membrane. Smaller amounts are delivered to other cells throughout the body.
2. The Tf-TfR complex is internalized, after which the iron is released into the cytosol, and apo-Tf is recycled intact to the plasma.
3. Iron utilization and storage
(a) Most iron (80–90%) is incorporated into hemoglobin, myoglobin, and cytochromes.
(b) A small amount is incorporated into nonheme enzymes (e.g., ribonucleotide reductase).
(c) The remaining iron is stored as ferritin and hemosiderin, mainly in cells that specialize in iron storage, i.e., macrophages and hepatocytes.

iii. *Iron excretion*
1. Iron normally is removed from the body only when cells are lost, especially epithelial cells of the GI tract, skin and renal tubules, and decidua from menstrual cycles. In some pathological conditions, e.g., GI blood loss or hemoglobinuria, iron is lost from the body in the form of heme in hemoglobin.
2. There is no physiologic mechanism for regulating iron excretion. Therefore, body iron balance in normal individuals is maintained by control of intestinal iron absorption. Derangements of this normal regulation of body iron balance occur in disorders of iron metabolism such as hemochromatosis and thalassemia major (Chap. 7).

Iron Depletion and Iron Deficiency

As iron stores become depleted, e.g., as a result of blood loss, three phases occur sequentially:

i. Initially, although iron stores become depleted, sufficient iron is still available for red cell production, and hemoglobin (Hb) levels remain normal. Tissue iron levels also remain normal, although the serum ferritin concentration begins to fall, indicating diminishing iron stores.
ii. As iron levels continue to decrease, tissue iron may start to become depleted.
1. At this stage, serum ferritin is low, as is the serum iron concentration; serum total iron-binding capacity (which is directly related to serum Tf concentration) is increased, and the percent saturation of transferrin with iron is decreased.
2. Hb and mean cell volume (MCV) remain within normal limits, but there may be a few hypochromic red cells visible on peripheral smear; the RDW may become elevated, as the first sign of developing iron deficiency to be apparent in the automated CBC.
iii. In the last phase, once iron stores have been fully depleted, there is no longer sufficient iron to maintain RBC production and, as losses continue, anemia results. RBCs become progressively hypochromic and microcytic. Other tissues may be affected by iron deficiency such as nails and tongue (see section "Clinical Manifestations," below).

Prevalence of Iron Deficiency

i. Iron deficiency affects approximately four to five billion individuals around the world, and up to two billion persons suffer from iron-deficiency anemia. In undeveloped countries, the cause is often nutritional deficiency or blood loss from parasitic infections.
ii. In developed western nations, the three most vulnerable populations are the following (with prevalences in the United States shown in parentheses [2, 3]):
1. Infants (14%) and preschool children (4%)
2. Women of childbearing age (9%)
3. Elderly (2–4% in males, 5–7% in females)

Causes of Iron Deficiency

A diagnosis of iron deficiency mandates a search for the cause; GI blood loss is the most common etiology, but other possibilities must be considered if a GI source is not identified.

Box 5.1 Causes of GI Blood Loss

1. *Esophagitis*
2. *Varices*
3. *Diaphragmatic (hiatal) hernia*
4. *Ulcers*
5. *Gastritis*
6. *Gastric antral vascular ectasia (GAVE syndrome, aka watermelon stomach)*
7. *Hemobilia*
8. *Arteriovenous malformations, hereditary hemorrhagic telangiectasia (Osler-Weber-Rendu syndrome)*
9. *Polyps*
10. *Tumors*
11. *Inflammatory bowel disease*
12. *Parasitic infection*
13. *Meckel diverticulum*
14. *Milk-induced enteropathy (in infants)*

Box 5.2 Examples of Other Causes of Iron Deficiency

- *Gynecologic (menses)*
- *Pregnancy*
- *Lactation*
- *Bladder neoplasms*
- *Epistaxis*
- *Blood donation*
- *Chronic intravascular hemolysis with hemoglobinuria*
- *Factitious anemia (self-inflicted bleeding/auto-phlebotomy)*
- *Pulmonary hemosiderosis*
- *Runner's anemia*

(A) *Causes of iron deficiency related to the GI tract*

i. In men and postmenopausal women with iron deficiency, GI blood loss must be considered the cause until proven otherwise. GI bleeding may be signaled by a history of melena or rectal bleeding, or a positive fecal occult blood test, although a negative test does not rule out a GI source.

ii. Colonoscopy and upper endoscopy are the first steps in evaluation and, if negative, should prompt a capsule endoscopy. Examples of possible sources of GI blood loss are listed in Box 5.1.

iii. Gastrectomy removes parietal cells that produce HCl required for the conversion of food iron from the Fe^{3+} state to the Fe^{2+} form for optimal absorption. Iron malabsorption due to gastric bypass surgery for obesity, which also bypasses the duodenum, is an increasingly common condition [4].

iv. Celiac disease is an important cause of iron malabsorption and iron deficiency.

v. In vegetarians, nonheme iron intake may not be adequate to supply needs, especially since this form of iron is less well absorbed than heme-iron found in meat. Iron deficiency is more common in vegans.

vi. Other possible causes of iron deficiency, attributable to losses of blood or iron, are listed in Box 5.2.

(B) *Causes of iron deficiency in infancy* [5]

i. Iron stores can be inadequate at birth as a result of maternal iron deficiency or prematurity, as half of the infant's iron stores accumulate in the last month of fetal life. Another mechanism is fetal-maternal hemorrhage.

ii. Inadequate dietary iron:

1. The need for iron is increased by a rapidly expanding red cell mass associated with growth. The growing child needs 0.5–1 mg of iron daily, and this amount cannot be supplied by breast milk alone in breast-fed infants.
2. Similarly, infants fed non-iron-fortified cow's milk formula have an increased risk of developing iron-deficiency anemia. In addition, whole cow's milk increases intestinal blood loss in infants. This may be the result of hypersensitivity or allergy; erosive gastritis or gastroduodenitis has been demonstrated endoscopically in some cases and may represent a source of bleeding [1].
3. Dietary iron frequently is inadequate in developing countries.

(C) *Causes of iron deficiency in women of childbearing age*

i. *Iron loss through menstruation*

1. Monthly blood loss in normal women ranges from 10 to 180 mL. However, the maximum iron content of a typical diet (20 mg daily, of which only about 10% is absorbed) can replace only the amount of iron in about 60 mL of monthly menstrual blood loss (corresponding to 60 mg of iron).
2. Thus, women whose monthly menstrual blood loss exceeds this amount are at risk of iron deficiency; the risk can be exacerbated by other factors such as decreased dietary iron intake or pregnancy, which can lead to iron deficiency and anemia.

ii. *Iron losses from pregnancy*

1. Iron loses of 500–700 mg are typical with pregnancy – 250 mg transferred to the fetus – and the remainder lost with the placenta and via hemorrhage.

2. Pregnant women thus need additional iron supplementation with 20–30 mg/day orally. Further iron is required to replace losses during lactation.

Clinical Manifestations

i. Patients can be asymptomatic or suffer from symptoms of anemia, including fatigue, weakness, pallor, palpitations, lightheadedness, headaches, tinnitus, or exertional dyspnea.
ii. Some patients have symptoms or signs related to the underlying cause of their iron deficiency, e.g.:
    1. GI symptoms/signs – abdominal pain or melena from a gastric or duodenal ulcer, hematochezia from a colonic polyp or cancer, positive fecal occult blood test
    2. Gynecological symptoms – heavy menses, cramping from uterine fibroids
iii. Certain manifestations are related to the direct effects of iron deficiency on tissues, including a number of classic symptoms and signs.

Symptoms

1. Dysphagia from esophageal webs/strictures (Plummer-Vinson syndrome) – a web of mucosa at the junction of the hypopharynx and esophagus
2. Pica – obsessive consumption of substances with no nutritional value, such as ice, starch, clay, paper, dirt, shoe laces
3. Restless leg syndrome – higher incidence in persons with iron deficiency

Signs

1. Glossitis – a smooth, waxy-appearing, red tongue, with atrophy of the papillae.
2. Angular cheilitis – ulcerations or fissures at the corners of the mouth.
3. Koilonychia – spooning of the nails, in which the nails are concave instead of convex.
4. Blue sclerae – although this occurs in osteogenesis imperfecta, the finding of blue sclerae in adults should alert the clinician to the possibility of iron deficiency.
5. Thrombocytosis – an elevated platelet count of unknown mechanism.

In addition, iron deficiency in children can lead to impaired psychomotor and mental development, not reversible by subsequent iron repletion.

Laboratory Diagnosis of Iron Deficiency

(A) *Complete blood count (CBC)*

The first abnormality in the CBC is an increased red cell distribution width (RDW, Chap. 4), followed by a decreasing MCV in the presence of anemia.

(B) *Peripheral blood smear* (Fig. 5.2)

In iron-deficiency anemia, the peripheral smear typically shows hypochromic, microcytic RBCs with some aniso- and poikilocytosis. Target cells can also be seen occasionally, and thrombocytosis may be present as well.

(C) *Serum ferritin*

i. The serum ferritin reflects total body storage iron, and a decrease in the ferritin concentration is the first laboratory sign of iron depletion.
ii. A serum ferritin below the laboratory's reference range (usually ~12 to 20 ug/L [ng/mL], depending on the laboratory) is diagnostic of iron deficiency.
iii. Serum ferritin is an "acute-phase reactant" and is often increased in the setting of inflammation (e.g., connective tissue diseases, infection, malignancy), hemolysis, hepatitis and other liver diseases, or bone marrow or splenic infarctions.
iv. Thus, the utility of serum ferritin in assessing iron stores is limited in the presence of inflammatory conditions (see section "The Anemia of Inflammation (Anemia of Chronic Disease)" below).

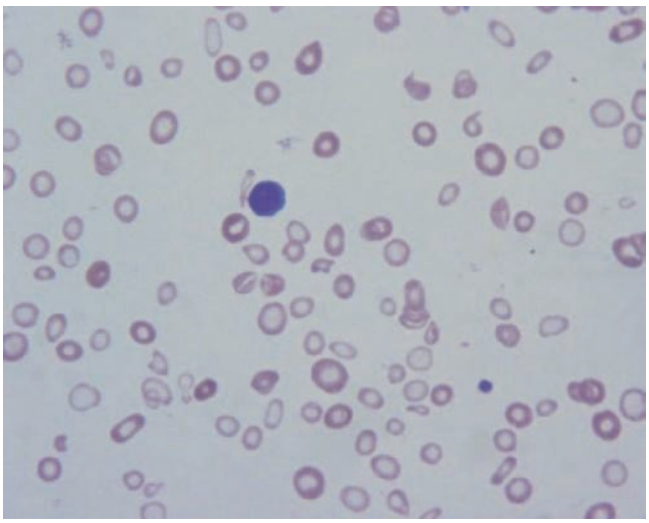

Fig. 5.2 Blood smear from a 16-year-old male who had a hemoglobin of 6 g/dL, MCV 55 fL, and a positive fecal occult blood test. He was found to have gastric polyps and was diagnosed with familial adenomatous polyposis. Note the very microcytic red cells in relation to the small lymphocyte in the center of the picture. Normal red cells should be about the same diameter as the nucleus of a small resting lymphocyte like the one pictured. The red cells are also hypochromic, with an increased area of central pallor and a narrow rim of hemoglobin

v. However, in patients with iron deficiency, ferritin usually does not rise above 200 μg/L (ng/mL) even if inflammation is present.

(D) *Iron studies*

i. Once storage iron is depleted, the serum iron level begins to fall, and TIBC increases.
ii. In an otherwise healthy person, a transferrin saturation of <10% with an elevated TIBC is diagnostic of iron deficiency.
iii. Serum iron and the percent saturation of transferrin are also low in inflammation.

(E) *Serum transferrin receptor*

The serum concentration of transferrin receptor (sTfR), a truncated form of TfR found normally in the circulation, is inversely related to body iron availability. Thus, the sTfR level is elevated in iron deficiency [6].

i. The sTfR level is not affected by inflammation, so sTfR can be helpful in the diagnosis of iron deficiency in patients with inflammatory conditions such as connective tissue disease, infection, or malignancy (see section "The Anemia of Inflammation (Anemia of Chronic Disease)" below).
ii. The serum sTfr concentration is also increased in the presence of hemolysis, and this must be borne in mind when interpreting the results.

(F) *Bone marrow examination*

Bone marrow aspiration is the gold standard for diagnosing iron deficiency. Normally, stainable iron can be seen in bone marrow macrophages. In addition, iron can be visualized in about one-third of normoblasts; these are called sideroblasts and are a normal finding, in contrast to abnormal ringed sideroblasts in conditions such as lead poisoning and sideroblastic anemia.

i. In iron deficiency, no stainable iron is visible in the aspirate.
ii. The aspirate is preferable to the bone marrow biopsy for assessing iron stores, because during the fixation processing required for biopsies, much of the stainable iron in the form of ferritin is solubilized.
iii. Bone marrow examination usually is not necessary solely to assess iron stores, as this generally can be accomplished with the aforementioned noninvasive tests.

Treatment of Iron Deficiency

i. The first principle of management is to identify and, if possible, correct the underlying cause of the iron deficiency, because this may be a sign of a potentially life-threatening lesion such as colon cancer.
ii. *Oral iron replacement is usually the treatment of choice*:

1. Many forms of medicinal iron are available, some better tolerated by patients than others, depending mainly on the amount of iron in each dose. Intestinal iron absorption is enhanced in iron deficiency, which facilitates iron repletion.
2. Oral iron preparations (e.g., iron salts such as ferrous sulfate) can cause GI upset (nausea and constipation). Dosing at 325 mg three times a day provides 65 mg of elemental iron with each dose. Other preparations contain 90 mg of elemental iron given twice daily. Oral iron also may cause very dark and tarry stools.
3. Although iron is best absorbed on an empty stomach (see 6., below), it is better tolerated with meals, and this approach is recommended at least initially. It is best to start with one dose a day and then gradually increase the frequency. If tolerated, doses may eventually be taken between meals.
4. Carbonyl iron, a bioavailable form of elemental iron that has long been used in food fortification, is well-absorbed and is less toxic than iron salts.
5. Recent studies suggest that iron may be better absorbed if taken less often, because hepcidin levels rise after an oral dose and can inhibit absorption from a subsequent oral dose [7]. That is, the iron-sensing mechanism in the liver responds to the rise in plasma iron in a feedback loop, resulting in increased hepcidin and decreased iron absorption.
6. Oral iron absorption can be impaired by certain foods and drink (tea, dairy products, grains) and by proton pump inhibitors. Absorption is enhanced by vitamin C.
7. An early indication of a response to iron repletion therapy is the appearance of polychromasia ("shift reticulocytes") in the peripheral blood smear, accompanied by a slight rise in the reticulocyte count, after 7–10 days.
8. With adequate iron repletion, hemoglobin values should rise by approximately 1 g/dL per week, which confirms the diagnosis. A failure of the hemoglobin to increase should lead to consideration of other factors such as:
   (a) Ongoing hemorrhage.
   (b) Nonadherence to the treatment regimen.
   (c) Iron malabsorption, as a result of gastrectomy, celiac disease, iron-refractory iron-deficiency anemia (IRIDA), or other causes [8]. Suspected malabsorption can be evaluated with an oral iron tolerance test.
   (d) The presence of another contributing cause of anemia such as folate deficiency.
   (e) Incorrect initial diagnosis.
9. Oral iron should be continued for 6 months to fully replete iron stores. This can be confirmed by measurement of serum ferritin concentration, demonstrating a return to normal levels.

10. Iron supplementation or iron replacement therapy should only be done under medical supervision, as chronic oral iron administration may be harmful.
    (a) Taking iron unnecessarily runs the risk of masking a bleeding lesion such as colon cancer that otherwise would be detected by the onset of anemia.
    (b) Susceptible persons (e.g., unrecognized hemochromatosis) may suffer iron overload.
    (c) Anemia should not be treated with iron unless a diagnosis of iron deficiency has been confirmed, although a brief trial of iron repletion to check for an increase in hemoglobin is reasonable if the diagnosis is uncertain (e.g., in the presence of concomitant inflammation).

iii. *Parenteral iron*
1. Parenteral iron is an alternative approach for patients with malabsorption or intolerance to oral iron. Some patients with ongoing GI iron loss (e.g., bleeding associated with small bowel angiodysplasia) cannot be maintained by oral replacement. Intravenous iron infusion is the preferred route.
2. Recently, new formulations have become available that enable total dose iron replacement in one to two sessions [9]. These include low-molecular-weight iron dextran, iron sucrose, ferumoxytol, ferric carboxymaltose, and iron isomaltoside. There is a small but significant risk of anaphylaxis (1–2%), and other infusion-related reactions can occur. These should be used with caution after an attempt to treat with oral iron has failed.

iv. *Blood transfusion*
1. Transfusion generally should be reserved for patients with severe anemia accompanied by clinical signs or symptoms of cardiovascular compromise.
2. Each unit of packed RBCs will raise the blood hemoglobin by about 1 g/dL.

The Anemia of Inflammation (Anemia of Chronic Disease)

The anemia of inflammation is characterized by a moderately decreased hemoglobin level (about 10–11 g/dL), generally without symptoms of anemia, and a normocytic, normochromic blood picture, although some patients (~30%) have a mild degree of microcytosis [10].

i. This condition must be differentiated from iron-deficiency anemia. A distinguishing feature is that the RDW is typically normal in the anemia of inflammation, in contrast to the elevated RDW in iron deficiency (Table 5.1). In addition, the C-reactive protein level is usually elevated with inflammation.
ii. The anemia of inflammation is commonly seen in patients with an ongoing infection, disseminated neoplasm, or connective tissue disease (e.g., rheumatoid arthritis). Correction of the anemia requires treatment of the underlying condition.
iii. Some other conditions are also complicated by inflammation, such as chronic kidney disease and hemodialysis.
iv. In inflammatory states, cytokines act to increase production of hepcidin (see section "Iron Metabolism" above), thereby limiting release of iron from stores in the RES and liver to the plasma and decreasing intestinal iron absorption (Fig. 5.1). This results in a decrease in serum iron concentration and the percent saturation of transferrin.
v. Such iron sequestration may represent a defense mechanism, i.e., a form of nutritional immunity, because microorganisms require iron for growth.
vi. TIBC levels are also low, in contrast with iron deficiency, in which the TIBC typically is elevated (Table 5.2).
vii. Serum ferritin, which can be increased out of proportion to iron stores in the presence of inflammation, may

Table 5.1 Typical changes in the complete blood count with iron-deficiency anemia and the anemia of inflammation

Condition	Degree of anemia	Mean corpuscular volume	Red cell distribution width	White blood cells	Platelets
Iron-deficiency anemia	Mild to severe	Decreased	Increased	Normal	Normal to increased
Inflammation	Mild	Normal to decreased	Normal	Normal to increased	Normal to increased

From: Rakel and Bope [11], with permission

Table 5.2 Typical changes in measures of iron status in iron deficiency and inflammation

Condition	Serum iron	Total iron-binding capacity	Transferrin saturation	Serum ferritin	Serum transferrin receptors
Iron deficiency	Decreased	Increased	Decreased	Decreased	Increased
Inflammation	Decreased	Decreased	Decreased	Normal to increased	Normal

From: Rakel and Bope [11], with permission

be normal or elevated (see section "Laboratory Diagnosis of Iron Deficiency," above).

viii. The sTfR level is normal; unlike serum ferritin and iron studies, this test is not affected by inflammation, so it can be used to diagnose iron deficiency even in the presence of inflammatory conditions (section "Laboratory Diagnosis of Iron Deficiency," above).

Other Microcytic Conditions (Box 5.3)

There are a number of other causes of microcytosis, including:

i. Hemoglobinopathies (see Chap. 7).
   - Thalassemia major: MCV low, accompanied by marked poikilo- and anisocytosis.
   - Thalassemia minor (β- or α-thalassemia carrier state): usually characterized by a combination of a decreased MCV, increased RBC count, and a normal RDW.
   - Other hemoglobinopathies (e.g., hemoglobin C, E). In hemoglobin C disease, precipitated Hgb C leads to small RBCs.

ii. Sideroblastic anemia
   - Congenital (X-linked or autosomal).
   - Acquired: microcytic anemias can occur in the context of several other conditions:
     - Heavy alcohol consumption
     - Lead poisoning
     - Pyridoxine (vitamin B6) deficiency
     - Use of certain medications (e.g., antituberculosis therapy with isoniazid, although severe anemia associated with the latter is relatively uncommon, given the regular concomitant administration of pyridoxine).

Box 5.3 Other Microcytic Anemias

Thalassemia major or minor (alpha or beta)
Other hemoglobinopathies (hemoglobin Lepore, hemoglobin C, hemoglobin E)
Sideroblastic anemia
Congenital (X-linked)
Acquired
Myelodysplastic syndromes[a]
Alcohol-induced
Lead poisoning
Vitamin B6 deficiency
Isoniazid
Anemia of chronic disease (section "The Anemia of Inflammation (Anemia of Chronic Disease)")

[a]More typically associated with macrocytosis, but some microcytosis can be seen in refractory anemia with ringed sideroblasts (RARS)

Summary

This chapter introduced the subject of iron metabolism, including the regulation of body iron distribution and intestinal iron absorption, followed by an explanation of the pathophysiology of iron depletion. The phases of the development of iron-deficiency anemia and its prevalence in different populations at risk are outlined. The clinical manifestations of iron deficiency and the resulting anemia are reviewed, along with a description of laboratory tests to diagnose the condition. The importance of determining the cause of the patient's iron deficiency and the approach to treatment is summarized. The final section focused on the anemia of inflammation and its differentiation from iron deficiency.

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Vitamin B_{12} (Cobalamin) and Folate Deficiency

6

Aśok C. Antony

General Considerations

(i) Folates and vitamin B_{12} (aka, cobalamin) participate in one-carbon metabolism (enzymatic reactions involving the transfer of one-carbon groups like methyl-, formyl-, methylene-, and formimino-) that are essential for pyrimidine and purine biosynthesis (including synthesis of three of four nucleotides of DNA).

(ii) Defective DNA synthesis in rapidly proliferating hematopoietic/gastrointestinal-epithelial/gonadal/fetal cells results in megaloblastic cells with DNA values that are "stuck" between 2N and 4N and therefore unable to divide, with adverse clinical consequences arising from affected hematopoietic/gastrointestinal-epithelial/gonadal/fetal cells.

(iii) Megaloblastic cells have "nuclear-cytoplasmic dissociation" (large "immature" nucleus with a relatively mature cytoplasm) (Fig. 6.1).

(iv) Deficiency of vitamin B_{12} or folates can present with megaloblastic anemia, but deficiency of vitamin B_{12} can present with neuropsychiatric syndromes.

(v) Correct vitamin replacement for either vitamin B_{12} or folate deficiency is essential.

(vi) The preexisting stores of these vitamins will dictate the speed with which overt deficiency develops.

Epidemiology

Vitamin B_{12} Nutrition

(i) Recommended daily allowance (RDA) of vitamin B_{12}:
  (a) Men/nonpregnant women = 2.4 μg.
  (b) Pregnant women = 2.6μg; lactating women = 2.8μg.
  (c) An even higher intake of 4–7 μg of cobalamin each day appears optimum for adequate cobalamin status.

(ii) Vitamin B_{12} is solely produced in nature by microorganisms; main dietary cobalamin is animal-source foods: There is no unfortified plant food that can consistently provide a sufficient amount of vitamin B_{12} in the diet:
  (a) Meat (>10 μg/100 g).
  (b) Fish, milk products, egg yolk (1–10 μg/100 g).
  (c) Nonvegetarian Western diets (5–7 μg/day).
  (d) Nonvegetarians (e.g., poverty-imposed near-vegetarians) with low animal-source food intake are also at risk.
  (e) Lacto-ovo vegetarians (<0.5 μg/day), vegans (<0.1 μg/day).

(iii) Vitamin B_{12} is exceptionally well stored (total stores = 2000–5000 μg vitamin B_{12}); 50% liver.

(iv) Daily loss = 1 μg, so elimination of dietary vitamin B_{12} intake takes 5–10 years to manifest clinically.

(v) Daily turnover (5–10 μg vitamin B_{12}/day) via efficient enterohepatic circulation with 75% reabsorption, so interruption (e.g., ileal resection) results in greater fecal losses; clinical presentation ~3–4 years.

(vi) Vitamin B_{12} resists high-temperature cooking but is unstable to light.

Folates Nutrition

(i) RDA folate:
  (a) Adult men/nonpregnant women = 400 μg.
  (b) Pregnant women = 600 μg for fetus and maternal tissues; lactating women = 500 μg.
  (c) Folates are synthesized by microorganisms, green-leafy vegetables/beans/fruit and animal-source foods.

(ii) A balanced Western diet prevents folate deficiency but is still generally considered inadequate during pregnancy for fetal folate requirements (hence the need for food fortification—see Tables 6.1 and 6.2).

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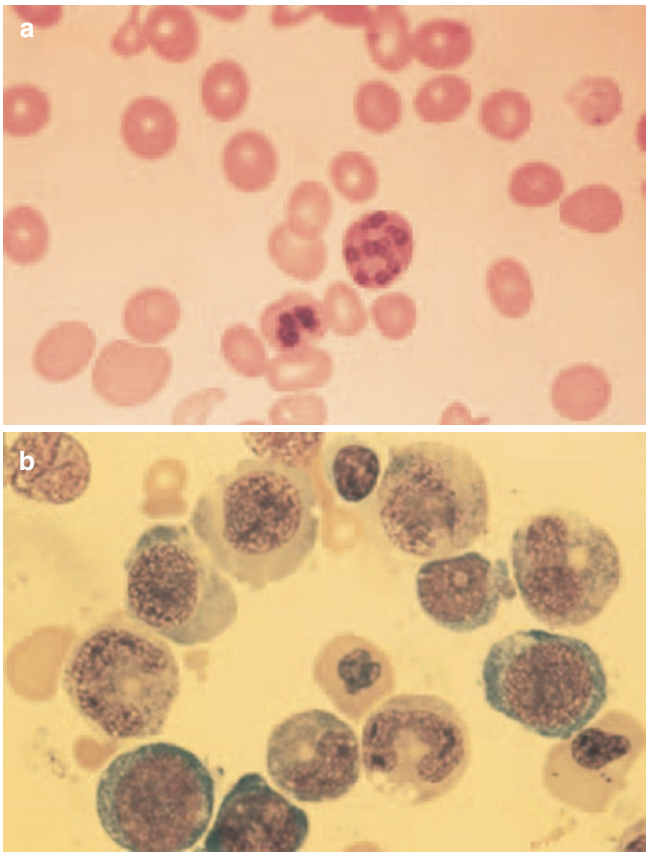

Fig. 6.1 Characteristic features of megaloblastic anemia in peripheral blood and bone marrow. (**a**) The peripheral blood has oval macrocytes and marked hypersegmentation of polymorphonuclear neutrophils. (**b**) A bone marrow aspirate shows megaloblastic normoblasts that exhibit nuclear-cytoplasmic dissociation or asynchrony (nuclear maturation lagging behind cytoplasmic maturation). Megaloblastic changes in the leukocyte series are shown by the "giant metamyelocytes." The orthochromatic normoblasts do not have pyknotic nuclei. (From: Antony AC. Megaloblastic Anemias, Chapter 170. In: Goldman L, Ausiello D, editors. Cecil Medicine. 23rd ed. Philadelphia: Saunders-Elsevier; 2008. p. 1231–41. Used with permission)

Table 6.1 Beneficial effects of folic acid and B_{12} therapy on non-hematopoietic systems[a]

1. *Using folic acid, vitamin B_{12}, pyridoxine supplementation*
Reduced hip fracture[b]
Reduced the progression of carotid intima media thickness[b] *(Surrogate marker of early subclinical arteriosclerosis)*
Reduction in age-related macular degeneration [b]
Slowing the rate of brain atrophy in elderly patients with mild cognitive impairment[a,b]
Prevention of cognitive decline (particularly in immediate and delayed memory performance) in community-dwelling older adults with depressive symptoms[a,b]
2. *Using folic acid supplementation*
Reduction in stroke[b]
Reduction in rate of cognitive decline among healthy elderly[b]
Reduction in age-related (sensorineural) hearing loss[b]
Reduction in recurrence of neural tube defects
Prevention of phenytoin-induced gingival hyperplasia
3. *Beneficial effects of folic acid fortification of food (population-based studies)*
Reduction in neural tube defects *(Anencephaly, spina bifida, encephalocele, meningocele, iniencephaly)*
Reduction in cleft lip with/without cleft palate
Reduction in severe congenital heart disease *(Endocardial cushion defects, conotruncal defects)*
Reduction in congenital pyloric stenosis, stenosis of pelvico-ureteric junction, limb-reduction defects, omphalocele
Reduction in stroke mortality
Decreased risk of low-birthweight and small-for-gestational-age babies

[a]Signifies Paper with Randomized Controlled Trial Data; GRADE A; see "Further Reading"
[b]Therapy is directed to lower homocysteine

Further Reading for this Table

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Table 6.2 Prophylaxis with vitamin B_{12} or folate

A. *Prophylaxis with vitamin B_{12}*
(a) Infants of vitamin B_{12}-deficient mothers
(b) Vegetarians, poverty-imposed near-vegetarians (5–10 μg vitamin B_{12}/day orally)
(c) Postgastrectomy (1 mg/day orally lifelong, usually with parenteral iron)
(d) Malabsorption of vitamin B_{12} from any mechanism (see Table 6.4) (1–2 mg vitamin B_{12}/day orally)
B. *Periconceptional supplementation of folate*[a]
(i) All normal women (400 μg folic acid/day) of childbearing age
(ii) Women with prior neural tube defect baby (need higher doses = 4 mg folic acid/day)
(iii) Women in childbearing age taking anticonvulsants (Dilantin/phenobarbital/carbamazepine) (1 mg folic acid/day)
C. *Folate supplementation given routinely*[a]
(a) Premature infants
(b) Lactating mothers; all those in resource-poor countries with a dietary history of poor intake of folate-rich foods
(c) Chronic hemolysis/myeloproliferative diseases (1 mg/day)
(d) To reduce toxicity of methotrexate (rheumatoid arthritis/psoriasis) (1 mg/day)
(e) Decrease in the risk for initiation or early development of colorectal cancer[a]
(f) Supplementation with folic acid protects against the development of colorectal neoplasia in high-risk patients with ulcerative colitis

[a]If folate is given to a patient with vitamin B_{12} deficiency, the patient can develop progressive neurologic damage

(iii) In developing countries folate intake often <1/2–1/3 of RDA.
(iv) Folates break down upon prolonged cooking (>15 min destroys 50–95% folate).

Vitamin B_{12} Physiology

The complex mechanism where several chaperone proteins *escort* small amounts of [precious] food-vitamin B_{12} through the intestine (Fig. 6.2) ensures maximal absorption and ultimate delivery into the cytoplasm of cells to participate as coenzymes in critical pathways. Main pathology is related to poor intake versus malabsorption (Fig. 6.2).

Normal Absorption and Transport

(i) Two coenzyme forms (deoxyadenosylcobalamin and methylcobalamin) in food must be released from bound proteins via peptic digestion at low gastric pH prior to absorption.
(ii) When released, vitamin B_{12} first binds salivary/gastric R-protein (haptocorrin).
(iii) After R-proteins digested by pancreatic proteases, vitamin B_{12} transferred to gastric intrinsic factor.
(iv) Intrinsic factor-vitamin B_{12} complex binds intrinsic factor-vitamin B_{12} receptors (aka cubam receptors) on ileal mucosal cells—"cubam" because receptor is composed of *cub*ilin-plus-*am*nionless proteins.
(v) Within enterocytes, vitamin B_{12} transferred to transcobalamin II and released into blood.
(vi) Transcobalamin II-vitamin B_{12} complex then binds to transcobalamin II receptors and internalized by receptor-mediated endocytosis.
(vii) Transcobalamin I binds and stores ~75% of vitamin B_{12} in blood.
(viii) Transcobalamin III "mops up" vitamin B_{12} analogs for hepatobiliary-to-fecal excretion.

Normal Cellular Processing

(i) >95% intracellular vitamin B_{12} bound to deoxyadenosylcobalamin or methylcobalamin.
(ii) Mitochondrial deoxyadenosylcobalamin is coenzyme for methylmalonyl-CoA mutase (converts methylmalonyl-CoA to succinyl-CoA so products of propionate metabolism (methylmalonyl-CoA) are easily metabolized).
(iii) Cytoplasmic methylcobalamin—a coenzyme for methionine synthase—catalyzes the transfer of methyl groups from methylcobalamin to homocysteine forming methionine; during reaction, methyl group of 5-methyl-tetrahydrofolate (methyl-THF) is donated to regenerate methylcobalamin, thereby forming THF that is essential to sustain one-carbon metabolism within cells.
(iv) When methionine is adenylated to *S*-adenosylmethionine, it can donate its methyl group for biologic methylation reactions involving >80 proteins, phospholipids, neurotransmitters, RNA, and DNA. Among these, DNA methylation is a major epigenetic mechanism that is central to the regulation of several cellular functions including gene silencing.

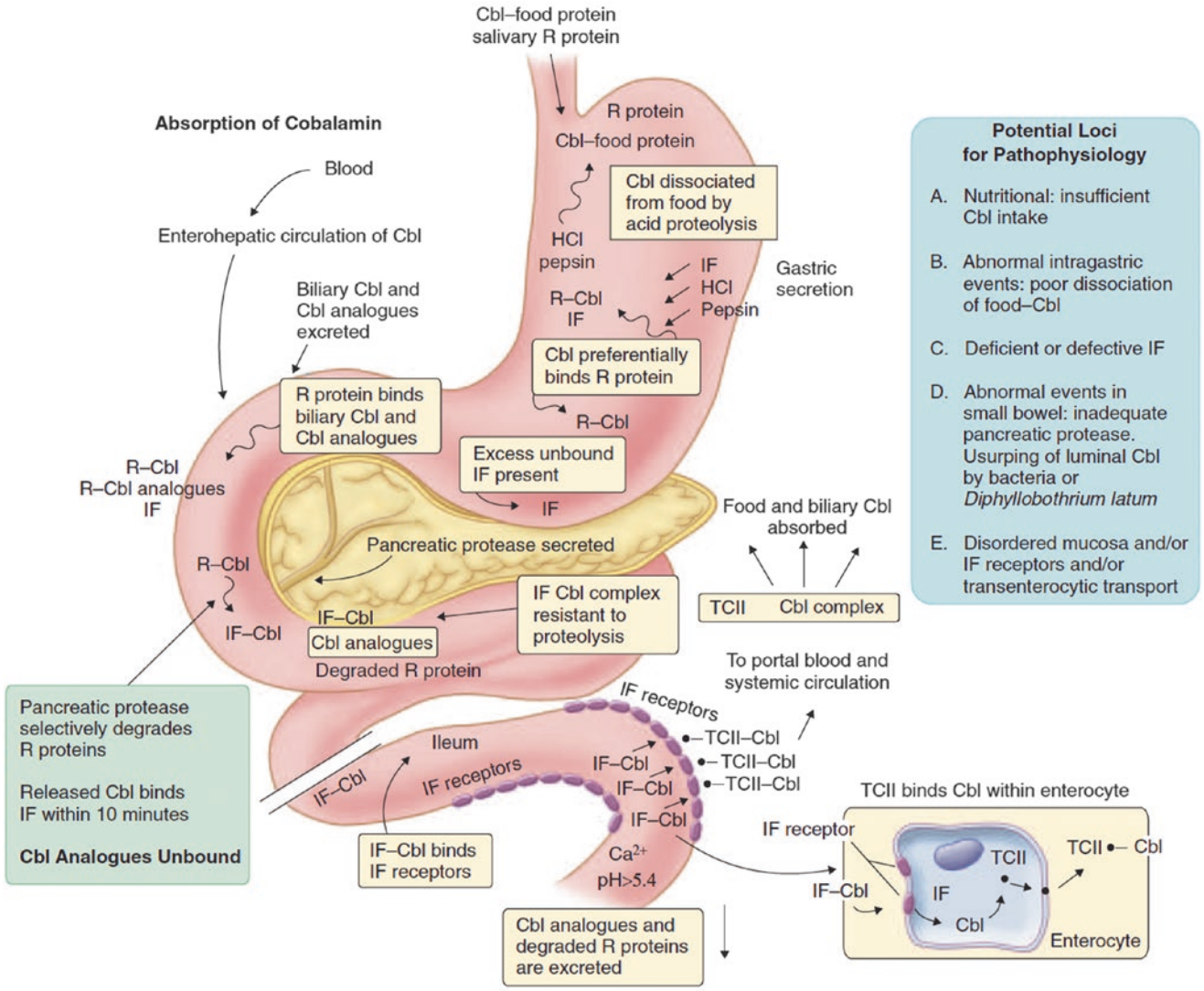

Fig. 6.2 Components and mechanism of vitamin B_{12} absorption with an indication of the locus for malabsorption. See text for details. *IF* intrinsic factor, *TCII* transcobalamin II. (Adapted from Antony AC. Megaloblastic anemias. In: Hoffman R, Benz Jr EJ, Shattil SJ, et al., editors. Hematology: basic principles and practice. 5th ed. Philadelphia., Churchill Livingstone: Elsevier; 2009. p. 491–524. Used with permission)

Pathogenesis of Vitamin B_{12} Deficiency (Table 6.3)

(i) *Nutritional vitamin B_{12} deficiency:* Vegetarianism and poverty-imposed near-vegetarianism is the commonest cause of nutritional vitamin B_{12} insufficiency worldwide in all age groups.

(a) The vast majority of those living in resource-limited countries subsist on a monotonous diet that is intrinsically low in animal-source foods. Although they are not strictly "vegetarians" since they do eat meat sparingly (e.g., once a week), these individuals are better classified as "near-vegetarians" and should also be supplemented with vitamin B_{12}. Near-vegetarians are found in large numbers among Chinese, Pakistani, Indian, most countries in Africa, Korean, and some Mediterranean populations.

(b) When mothers do not consume sufficient amounts of animal-source foods, they themselves are at risk for nutritional cobalamin deficiency, and their infants will have smaller stores of the vitamin at birth.

(ii) *Inadequate dissociation of vitamin B_{12} from food protein:* Failure to release dietary vitamin B_{12} from food protein (food-vitamin B_{12} malabsorption) can be found in 30–50% elderly with low vitamin B_{12} status (~ten-fold more common than pernicious anemia)

(iii) *Absent intrinsic factor secretion:*

(a) Total gastrectomy, pernicious anemia.

(b) Partial gastrectomy predisposes to multifactorial vitamin B_{12} deficiency (decreased intrinsic factor, hypochlorhydria, bacterial overgrowth of vitamin B_{12}-consuming organisms).

(c) Pernicious anemia = autoimmune destruction of gastric fundus/body leads to gastric atrophy, absent

Table 6.3 Classification of vitamin B_{12} and folate deficiency

1. *Vitamin B_{12} deficiency*
A. *Nutritional vitamin B_{12} deficiency* ***(insufficient vitamin B_{12} intake)***
(i) Vegetarians, poverty-imposed near-vegetarians
(ii) Breast-fed infants of mothers with pernicious anemia
B. *Abnormal intragastric events* ***(inadequate proteolysis of food-vitamin B_{12})***
(i) Atrophic gastritis with hypochlorhydria
(ii) Proton-pump inhibitors, H_2 blockers
C. *Loss/atrophy of gastric oxyntic mucosa* ***(deficient intrinsic factor molecules)***
(i) Total or partial gastrectomy or caustic destruction (lye)
(ii) Adult and juvenile pernicious anemia
D. *Abnormal events in the small bowel lumen*
(a) Inadequate pancreatic protease (*R-protein-vitamin B_{12} not degraded, vitamin B_{12} not transferred to intrinsic factor*)
(i) Insufficient pancreatic protease—pancreatic insufficiency
(ii) Inactivation of pancreatic protease—Zollinger-Ellison syndrome
(b) Usurping of luminal vitamin B_{12} (*inadequate binding of vitamin B_{12} to intrinsic factor*)
(i) By bacteria—stasis syndromes (blind loops, pouches of diverticulosis, strictures, fistulas, anastomosis), impaired bowel motility (scleroderma), hypogammaglobulinemia
(ii) By *Diphyllobothrium latum* (fish tapeworm)
E. *Disorders of ileal mucosa/intrinsic factor-vitamin B_{12} receptors* ***(intrinsic factor-vitamin B_{12} not bound to intrinsic factor-vitamin B_{12} receptors [aka, cubam receptors])***
(i) Diminished or absent cubam receptors—ileal bypass/resection/fistula
(ii) Abnormal mucosal architecture/function—tropical/nontropical sprue, Crohn's disease, tuberculous ileitis, amyloidosis
(iii) Cubam receptor defects—Imerslund-Gräsbeck syndrome, hereditary megaloblastic anemia
(iv) Drug effects—metformin, cholestyramine, colchicine, neomycin
F. *Disorders of plasma vitamin B_{12} transport* ***(TCII-vitamin B_{12} not delivered to TCII receptors)***—congenital TCII deficiency, defective binding of TCII-vitamin B_{12} to TCII receptors (rare)
G. *Metabolic disorders* ***(vitamin B_{12} not used by cell)—****multiple inborn enzyme errors (rare)*
H. *Acquired disorders* ***(vitamin B_{12} functionally inactivated by irreversible oxidation)***—nitrous oxide (N_2O) inhalation
2. *Folate deficiency*
A. *Nutritional causes*
(1) Decreased dietary intake—poverty and famine, institutionalized individuals (psychiatric/nursing homes)/chronic debilitating disease, prolonged feeding of infants with goat's milk, special slimming diets or food fads *(folate-rich foods not consumed),* cultural/ethnic cooking techniques *(food folate destroyed)*
(2) Decreased diet and increased requirements
(a) Physiologic—pregnancy and lactation, prematurity, hyperemesis gravidarum, infancy
(b) Pathologic
(i) *Intrinsic hematologic diseases* involving hemolysis with compensatory erythropoiesis, abnormal hematopoiesis, or bone marrow infiltration with malignant disease
(ii) *Dermatologic disease*—extensive psoriasis
B. *Folate malabsorption*
(1) With normal intestinal mucosa
(a) Drugs: sulfasalazine, pyrimethamine, proton-pump inhibitors (via *inhibition of the proton-coupled folate transporters*)
(b) Hereditary folate malabsorption *(mutations in proton-coupled folate transporters)* (rare)
(2) With mucosal abnormalities—tropical and nontropical sprue, regional enteritis
C. *Inadequate cellular utilization*
(1) Defective CSF folate transport—cerebral folate deficiency (*autoantibodies to folate receptors*)
(2) Hereditary enzyme deficiencies involving folate (rare)
D. *Drugs (multiple effects on folate metabolism)*—Folate antagonists (methotrexate), alcohol, sulfasalazine, triamterene, pyrimethamine, trimethoprim-sulfamethoxazole, diphenylhydantoin, barbiturates
3. *Miscellaneous megaloblastic anemias not caused by vitamin B_{12} or folate deficiency*
A. *Congenital disorders of DNA synthesis, orotic aciduria, Lesch-Nyhan syndrome, congenital dyserythropoietic anemia*
B. *Acquired disorders of DNA synthesis*
(i) Deficiency—thiamine-responsive megaloblastic anemia *(thiamine transporter 1 mutation)*
(ii) Malignancy—erythroleukemia
(iii) All antineoplastic drugs that inhibit DNA synthesis *(antinucleosides used against human immunodeficiency and other viruses)*, toxic—alcohol

intrinsic factor, and achlorhydria which lead to vitamin B_{12} malabsorption and then deficiency.

- Anti-intrinsic factor antibodies (highly specific for pernicious anemia) ~60% serum; ~75% gastric juice. Combining anti-intrinsic factor antibodies (37% sensitivity; 100% specificity) with newer-generation anti-parietal cell antibodies (91% sensitivity; 90% specificity) can significantly increase the diagnostic performance for pernicious anemia, yielding overall 73% sensitivity while maintaining 100% specificity.
- Average age for pernicious anemia ~60 years but all ages and races affected (S. California study, ~2% >60 years had undiagnosed subclinical pernicious anemia, but ~4% of white/African-American women had pernicious anemia).
- ~30% have positive family history.
- Positive associations with autoimmune diseases (Graves' disease, Hashimoto's thyroiditis, vitiligo, Addison's disease, hypoparathyroidism, myasthenia gravis, type 1 diabetes mellitus, hypogammaglobulinemia).

(iv) *Abnormal events precluding absorption of vitamin B_{12}*
   (a) Massive gastric hypersecretion (gastrinoma/Zollinger-Ellison syndrome) inactivates pancreatic protease and interferes with ileal absorption at luminal pH <5.4.
   (b) Overgrowth by vitamin B_{12}-consuming bacteria or fish tapeworms can usurp vitamin B_{12} before it binds intrinsic factor.
   (c) Jejunal adult fish tapeworms *Diphyllobothrium latum* enter as plerocercoid larvae embedded in *sushi* (so infected *sushi* = Trojan fish!); (Note: fish tapeworms are 10 m long; 10^6 eggs released/worm/day; worm life span = 20 years!).

(v) *Disorders of the intrinsic factor receptors or mucosa*
   (a) Terminal 2 ft of ileum have greatest density of cubam receptors, so removal/bypass/dysfunction leads to severe vitamin B_{12} malabsorption.
   (b) Metformin interferes with vitamin B_{12} absorption in 1/3, reversed by calcium (1.2 g/day).

(vi) *Acquired vitamin B_{12} deficiency*
   (a) Nitrous oxide (N_2O) irreversibly inactivates the vitamin B_{12} molecule leading to intracellular functional folate deficiency, reversed by intravenous 5-formyl-THF (leucovorin).
   (b) Patients with marginal vitamin B_{12} stores are at risk during prolonged surgery with N_2O anesthesia.
   (c) Chronic intermittent N_2O exposure presents with neuromyelopathic manifestations.

Folate Physiology

(i) *Normal folate absorption*
   (a) ~50% food folate (polyglutamylated) bioavailable after hydrolysis to monoglutamates.
   (b) ~85% folic acid (in fortified food/supplements) bioavailable.
   (c) Factors that increase bioavailability of folates (pureed food, ascorbate); decreased bioavailability of folate (organic acid/orange juice, sulfasalazine, ethanol)
   (d) Luminal surface proton-coupled folate-transporter protein (duodenum/jejunum) facilitates folate transport into enterocytes and then released into plasma as methyl-THF.
   (e) Serum folate level is determined by dietary folate intake and an efficient enterohepatic circulation.

(ii) *Normal folate transport:* Rapid cellular uptake of methyl-THF/folic acid by two mechanisms.
   (a) High-affinity surface folate receptors bind and internalize physiologically-relevant methyl-THF, folic acid, and some newer antifolates with high affinity. After folate receptor-mediated endocytosis, proton-coupled folate transporter exports folate from acidified endosomes into cytoplasm of cells, across placenta to fetus, and across choroid plexus into CSF.
   (b) Reduced folate carriers have low affinity but high capacity for methyl-THF, methotrexate, and folinic acid (folic acid uptake is poor).
   (c) Passive diffusion across all membranes operates at supraphysiologic folate concentrations.

(iii) *Intracellular metabolism and vitamin B_{12}-folate interactions*
   (a) Methyl-THF must be converted to THF (via methionine synthase) so THF can be polyglutamated and retained for one-carbon metabolism. Vitamin B_{12} is a cofactor for this reaction.
   (b) THF converted to 10-formyl-THF for de novo biosynthesis of purines and to methylene-THF.
   (c) With inactivation of methionine synthase during vitamin B_{12} deficiency, methyl-THF is not polyglutamylated and therefore leaks out of cell, resulting in intracellular THF deficiency.
   (d) Methylene-THF used by thymidylate synthase to synthesize thymidine and DNA or after conversion to methyl-THF (via methylene-THF reductase) for methionine synthase.
   (e) Vitamin B_{12} deficiency can respond to replacement with folic acid because it can be converted to THF (via dihydrofolate reductase); alternatively, 5-formyl-THF (folinic acid) bypasses methionine synthase and can be converted to methylene-THF for DNA synthesis.
   (f) When methionine synthase is inhibited during either vitamin B_{12} or folate deficiency, there is a functional folate deficiency with an intracellular buildup of 5-methyltetrahydrofolate that leaks out of cells; when vitamin B_{12} deficiency is prolonged,

this can lead to a true folate deficiency. In addition, there is accumulation of the thiol amino acid, homocysteine, which can have multiple deleterious effects on the body through a variety of molecular and biochemical pathways. This explains the observed beneficial effects noted following therapy with supplemental vitamin B_{12} and/or folate deficiency to lower hyperhomocysteinemia (Table 6.1). Patients with vitamin B_{12} deficiency (pernicious anemia and/or nutritional vitamin B_{12} deficiency) can have normal to high serum folate levels (*that can mask an associated mild to moderate folate deficiency*) and high serum homocysteine levels.

(g) Because another vitamin B_{12}-dependent enzyme cannot function during vitamin B_{12} deficiency, there is accumulation of methylmalonic acid within cells which also leaks out into the serum.

(iv) *Consequences of vitamin B_{12}-folate deficiency*

(a) During folate deficiency, accumulated cellular homocysteine covalently binds hnRNP-E1 (a mRNA-binding protein) which can then bind folate receptor mRNA to upregulate folate receptors; here, hnRNP-E1 functions as a sensor for cellular folate deficiency.

(b) Homocysteinylated hnRNP-E1 also binds to and activates a family of unrelated RNAs that are recognized by common signature sequences (i.e., as members of a posttranscriptional RNA operon); this results in over- and under-expression of scores of mRNA-derived cellular proteins, many of which contribute to the observed features of megaloblastic anemia (reduced cell proliferation and differentiation and apoptosis).

(c) Folate deficiency leads to fragmentation of DNA in both common and rare fragile sites, which leads to genomic instability that renders tissues more susceptible to cancer; folate deficiency also facilitates genomic integration of HPV16 DNA that can trigger pathways to HPV16-induced carcinogenesis.

(v) *Renal conservation*

(a) Folate receptors on proximal renal tubular cells bind and return luminal folate back to blood.

Pathogenesis of Folate Deficiency (Key Points) (Table 6.3)

(i) Folate deficiency arises from *decreased supply* (reduced intake, absorption, transport, or utilization) or *increased requirements* (from metabolic consumption, destruction, or excretion).

(ii) One individual may have multiple causes for folate deficiency.

Nutritional Causes (Decreased Intake or Increased Requirements)

(i) Factors predisposing to reduced stores: poverty, seasonal reduction in folate-rich food, cultural/ethnic destructive cooking techniques/diets intrinsically poor in folates, anorexia (chronic illness), hemolysis (malaria), exfoliative skin diseases, alcoholism, pregnancy/lactation.

(ii) Body stores of folate adequate for ~4 months only.

(iii) Nutritional folate insufficiency (poor dietary intake) is the commonest cause worldwide in all age groups, with women and children in developing countries at highest risk.

(iv) Food fortification with folic acid has almost entirely eliminated folate deficiency in the USA, but socially isolated/infirm individuals subsisting on imbalanced diets are still at risk.

Pregnancy and Infancy

(i) Short inter-pregnancy intervals and twin pregnancies predispose to folate deficiency.

(ii) Inadequate maternal folate predisposes to premature, low-birthweight infants and predominantly midline fetal developmental abnormalities (neural tube defects/cleft lip/cleft palate/endocardial cushion defects), including early childhood behavioral abnormalities (Table 6.3).

(iii) Folate receptor autoantibodies in some mothers linked to recurrent neural tube defects.

(iv) Cerebral folate deficiency:

(a) Caused either by congenital mutations or autoantibodies to folate receptors can perturb folate receptor-mediated folate transport into the brain (via the choroid plexus) and lead to cerebral folate deficiency.

(b) These autoantibodies to human folate receptors develop against closely related folate-binding proteins found in cow's milk that share common epitopes.

(c) Neonatal presentation (~6 months): agitation/insomnia, deceleration of head growth, psychomotor retardation, hypotonia/ataxia, spasticity, dyskinesias, epilepsy, and even autistic features.

(d) Affected children respond to high-dose folinic acid *and* a bovine milk-free diet.

(v) Hereditary folate malabsorption:

(a) Mutation in proton-coupled folate transporter (intestine/choroid plexus).

(b) Presents with megaloblastic anemia, chronic diarrhea, neurologic abnormalities (seizures/mental retardation) usually in childhood.

(c) Responds to high-dose parenteral folinic acid.

Tropical and Nontropical (Celiac) Sprue

(i) Tropical sprue responds to a 4–6-month course oral folic acid (5 mg/day) plus tetracycline 250 mg QID in ~60% of patients.
(ii) Chronic tropical sprue (>3 years) associated with vitamin B_{12} malabsorption-plus-iron/pyridoxine/thiamine deficiencies.

Drugs

(i) Trimethoprim, pyrimethamine, or methotrexate inhibits dihydrofolate reductase.
(ii) Sulfasalazine inhibits proton-coupled folate transporters and induces Heinz body hemolytic anemia.
(iii) Pyrimethamine/proton-pump inhibitors inhibit proton-coupled folate transporters.
(iv) Oral contraceptives increase folate catabolism.
(v) Anticonvulsants reduce absorption and induce microsomal liver enzymes.
(vi) Antineoplastics and antiretroviral antinucleosides (azidothymidine) perturb DNA synthesis independently of folate/vitamin B_{12}.

Clinical Presentations of Folate/Vitamin B_{12} Deficiency (see Table 6.1)

(i) Vitamin B_{12} deficiency develops insidiously, whereas folate-deficient patients are usually poorly nourished with multiple vitamin deficiencies.
(ii) The underlying condition that predisposed to folate deficiency will have occurred ~6 months previously and can dominate the clinical picture.
(iii) In developing countries nutritional vitamin B_{12} deficiency manifests as florid pancytopenia, mild hepatosplenomegaly, fever, and thrombocytopenia, with neuropsychiatric manifestations developing later.
(iv) Among the affluent in developing/developed countries, vitamin B_{12}-related neuropsychiatric disease may be found with only mild to moderate anemia (~25% to 50% have normal hematocrit/MCV; inverse correlation between hematocrit and neurologic disease).
(v) Megaloblastosis with intramedullary hemolysis gives pallor and jaundice (lemon-tint icterus).

Spectrum of Clinical Presentations of Folate/Vitamin B_{12} Deficiency (Tables 6.1 and 6.2)

(i) Clinical findings may be dominated by the underlying condition that caused deficiency of vitamin B_{12} or folate (Table 6.1).
(ii) Folate and vitamin B_{12} deficiency can present with following "systems" abnormalities:
  (a) Hematologic pancytopenia with megaloblastic bone marrow.
  (b) Cardiopulmonary congestive heart failure secondary to anemia.
  (c) Gastrointestinal glossitis with a smooth (depapillated), beefy-red tongue.
  (d) Dermatologic hyperpigmentation of the skin and premature graying.
  (e) Reproductive infertility, sterility, megaloblastic cervical epithelium mimicking dysplasia.
  (f) Psychiatric flat affect.
  (g) Neurological presentations suggest associated vitamin B_{12} deficiency or additional systemic disease (e.g., alcoholism with folate + thiamine deficiency).
(iii) Vitamin B_{12} deficiency can present with either of the following:
  (a) Dominant hematologic manifestations (like folate deficiency).
  (b) Neurological disease: widespread, patchy demyelination expressed clinically as cerebral abnormalities and subacute combined degeneration of the spinal cord. Vegetarians with vitamin B_{12} deficiency-related neuropathy can present with cognitive impairment.
  (c) Dorsal columns (thoracic segments) with contiguous involvement of corticospinal, spinothalamic, spinocerebellar tracts, and peripheral neuropathy.
  (d) Paresthesias are early findings with loss of position sense in index toes (before great toe); diminished vibration sense (256 cps tuning fork); positive Romberg/Lhermitte signs; later bladder-bowel incontinence, cranial nerve paresis, dementia/psychoses/mood disturbances.
(iv) Patients with chronic hyperhomocysteinemia (long-standing vitamin B_{12}/folate deficiency) may present with:
  (a) Occlusive vascular disease (small vessel cerebrovascular disease-related strokes, end-stage renal failure, thromboangiitis obliterans, aortic atherosclerosis, arterial and venous thromboembolism)
  (b) Occlusive placental vascular disease-related pregnancy complications (preeclampsia, placental abruption/infarctions, early recurrent miscarriage) or poor pregnancy outcomes (preterm delivery, neural tube defects, congenital heart defects, and intrauterine growth retardation)
  (c) Osteopenia with fractures
(v) Clinical conditions benefitted by vitamin supplementation (Tables 6.1 and 6.2) imply that patients with long-standing untreated deficiencies can present with:
  (a) Hip fractures and age-related macular degeneration
  (b) Strokes
  (c) Poor cognitive function in adults
  (d) Reduced low-frequency hearing in older adults

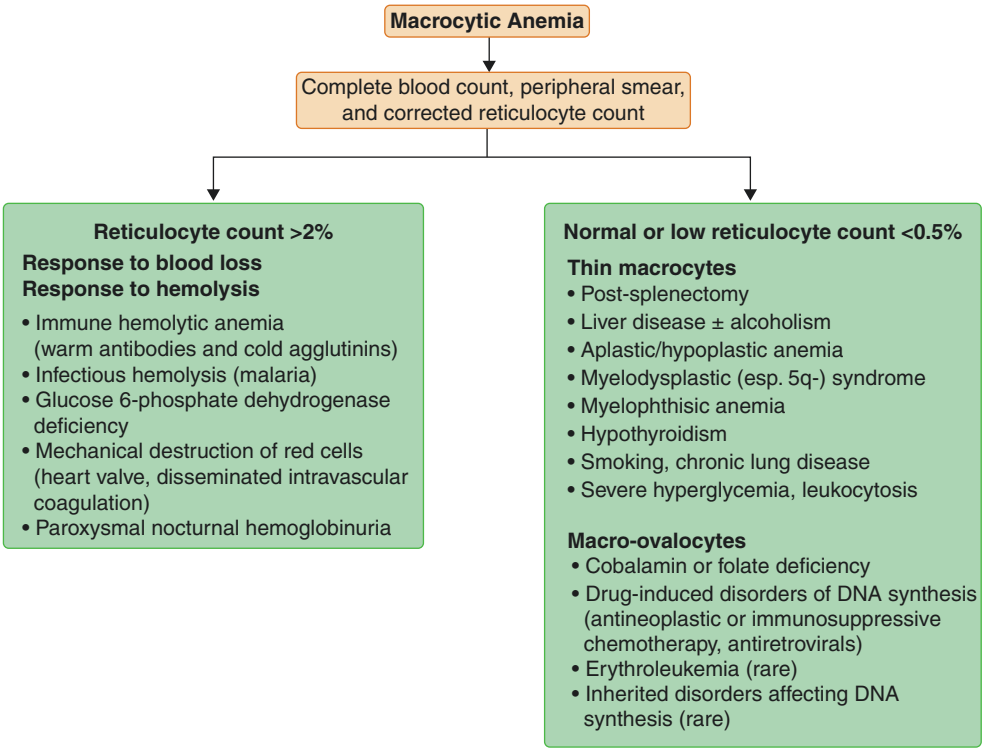

Fig. 6.3 Algorithm for evaluation of a patient with macrocytosis (Thin macrocytes have >1/3 central pallor; although deficiencies of vitamin B12 and folate are only two of the myriad of causes of macrocytosis, they become increasingly more likely as the MCV increases beyond 105 fl). (Adapted from: Antony AC. Megaloblastic Anemias, Chapter 170. In: Goldman L, Ausiello D, editors. Cecil Medicine. 23rd ed. Philadelphia: Saunders-Elsevier; 2008. p. 1231–41. Used with permission)

(vi) Children of mothers with low vitamin B_{12}/folate during pregnancy can present with:
  (a) Reduced neurocognitive performance (low-maternal vitamin B_{12})
  (b) Behavioral abnormalities (hyperactivity/inattention, peer problems in childhood) (low-maternal folate).

Diagnostic Approach to the Patient

There are three sequential steps:

(i) *Recognize* underlying megaloblastic anemia (Figs. 6.1 and 6.3) or potential vitamin B_{12}-related neurological presentation.
(ii) *Distinguish* whether folate, vitamin B_{12}, or both deficiencies resulted in clinical picture (Table 6.4).
(iii) *Identify the underlying disease* and likely mechanism causing deficiency (see Table 6.1 for identity of usual suspects).

Laboratory Tests (Fig. 6.1 and Table 6.4)

Megaloblastosis

The complete blood count (pancytopenia varying degree):

(i) Anemia—macrocytic anemia with steadily increasing MCV over time (hemoglobin ~5 g/dl sometimes)
(ii) Reticulocytopenia
(iii) Neutropenia and thrombocytopenia (rarely neutrophils <1000/μL or platelets <50,000/μL)
(iv) Intramedullary hemolysis (increased serum LDH and bilirubin; decreased haptoglobin)

Peripheral Smear (Fig. 6.1)

(i) Macro-ovalocytes (~14 mm).
(ii) Hypersegmented polymorphonuclear neutrophils (5% with five lobes or 1% with six lobes).
(iii) Megathrombocytes.
(iv) Nucleated RBC (rare).
(v) Megaloblastic anemia can be masked by associated iron deficiency/thalassemia (clue: look for hypersegmented polymorphonuclear leukocytes in smear).

Vitamin B_{12} and Folate Levels (Table 6.4)

(i) Suggestive clinical information improves pretest probability of results of serum vitamin B_{12}/folate levels.
(ii) Therefore these tests are only useful to confirm a high index of suspicion that patient has either folate or vitamin B_{12} deficiency.
(iii) If folate/vitamin B_{12} test results are borderline normal or ambiguous, proceed to use metabolite levels (Table 6.4).
(iv) *Caveat*: Any clinical presentation which strongly suggests vitamin B_{12} deficiency should always lead to a

Table 6.4 Stepwise approach to the diagnosis of vitamin B_{12} and folate deficiencies

Megaloblastic anemia or neurologic-psychiatric manifestations consistent with vitamin B_{12} deficiency plus *Test results on serum vitamin B_{12} and serum folate*			
Vitamin B_{12}[a] (pg/mL)	*Folate[b] (ng/mL)*	*Provisional diagnosis*	*Proceed with metabolites?[c]*
>300	>4	Vitamin B_{12}/folate deficiency is unlikely	No
<200	>4	*Consistent* with vitamin B_{12} deficiency	No
200–300	>4	*Rule out* vitamin B_{12} deficiency	Yes
>300	<2	*Consistent* with folate deficiency	No
<200	<2	*Consistent* with (i) combined vitamin B_{12} *plus* folate deficiency or (ii) isolated folate deficiency	Yes
>300	2–4	*Consistent* with (i) folate deficiency or (ii) an anemia unrelated to vitamin deficiency	Yes
Test results on metabolites: serum methylmalonic acid and total homocysteine			
Methylmalonic acid (normal = 70–270 nM)	*Total homocysteine (normal = 5–14 μM)*	*Diagnosis*	
Increased	Increased	Vitamin B_{12} deficiency confirmed; folate deficiency still possible (i.e., combined vitamin B_{12} plus folate deficiency possible)	
Normal	Increased	Folate deficiency is likely; <5% may have vitamin B_{12} deficiency	
Normal	Normal	Vitamin B_{12} and folate deficiencies are excluded	

[a]Serum vitamin B_{12} levels: abnormally low, less than 200 pg/mL; clinically relevant low to normal range, 200–300 pg/mL
[b]Serum folate levels: abnormally low, less than 2 ng/mL; clinically relevant low to normal range, 2–4 nm/mL
[c]Any frozen-over sample from serum folate/vitamin B_{12} determination can be subjected to metabolite tests
Adapted from Antony AC. Megaloblastic anemias. In: Hoffman R, Benz Jr EJ, Shattil SJ, et al., editors. Hematology: basic principles and practice. 4th ed. Philadelphia: Churchill Livingstone; 2005. p. 519–56. Used with permission

therapeutic trial (with vitamin B_{12} replacement) even if the laboratory assay for serum vitamin B_{12} is non-concordant (test kits are sometimes faulty).

(v) *Caveat*: Release of the 30-fold level of excess folate from infected erythroid precursors, reticulocytes, and mature erythrocytes during hemolysis from any cause (malaria, glucose 6-phosphate dehydrogenase deficiency, thalassemia, sickle cell disease) raises serum folate levels *and can mask an underlying tissue folate deficiency*.

(vi) Misleading elevations of the serum folate level in vitamin B_{12} deficiency and/or malaria are consistently common in resource-limited settings. In such settings, the serum folate concentration can range from normal to high, leading to serious *underestimation* of tissue folate status (i.e., altogether missing the diagnosis of severe folate deficiency). This predictable masking of tissue folate depletion argues against the use of serum tests for folate deficiency in this clinical setting. Instead, assessing the intake of folate-rich foods in the diet is a better method to assess folate status.

Metabolite Levels: Serum Homocysteine and Methylmalonic Acid (MMA) (Table 6.4)

(i) Gold standards for confirming vitamin B_{12} deficiency.

(ii) Serum homocysteine and MMA rise proportionate to severity of deficiency (Table 6.4).

(iii) Serum MMA elevated in >95% with clinically confirmed vitamin B_{12} deficiency (median ~3500 nM).

(iv) Serum homocysteine elevated in both vitamin B_{12} deficiency (median ~70 μM) and folate deficiency (median 50 μM).

(v) Both homocysteine and MMA rise with dehydration or renal failure; colonic bacteria contribute propionic acid that elevates MMA (metronidazole reduces this contribution).

(vi) *Caveat*: Increase in both metabolites cannot differentiate between pure vitamin B_{12} deficiency and combined vitamin B_{12}-plus-folate deficiency.

(vii) Elevated metabolites return to normal in a week with appropriate vitamin replacement.

Bone Marrow Examination for Rapid Diagnosis (1 h) of Megaloblastosis

(i) Trilinear hyperplasia with (ineffective) megaloblastic hematopoiesis

(ii) Megaloblastic orthochromatic megaloblasts containing finely stippled, reticular, immature non-pyknotic nuclei (which contrasts with clumped chromatin of orthochromatic normoblasts) and hemoglobinized cytoplasm

(iii) Megaloblastic leukopoiesis pathognomonic giant (20–30 μm) metamyelocytes, hypersegmented polymorphonuclear leukocytes

(iv) Megaloblastic megakaryocytes with complex hypersegmentation

Determining the Cause of Vitamin Deficiency

(i) Because the Schilling test (used to define the mechanism of malabsorption of vitamin $B1_2$) is not currently available in the USA, we must rely heavily on history/physical examination and judicious use of confirmatory laboratory tests:
  (a) Combined use of serum anti-intrinsic factor and newer-generation anti-parietal cell antibodies has 73% sensitivity while maintaining 100% specificity for pernicious anemia.
  (b) Stool for ova.
  (c) Serum IgA anti-tissue transglutaminase antibodies, lipase, gastrin.
  (d) Intestinal biopsy.
  (e) Radiographic contrast studies (stasis/strictures/fistulas).

(ii) For young patients (gastric juice intrinsic factor/achlorhydria, DNA for mutations in cubam receptor/proton-coupled folate transporters, or serum antifolate receptor antibodies)

Treatment

(i) In decompensated patient, draw blood for folate/vitamin B_{12}/metabolite levels (consider urgent confirmatory bone marrow) and transfuse patient with one unit packed red cells *slowly* under aggressive diuretic coverage to avoid acute pulmonary edema.

(ii) Administer full-dose folate and vitamin B_{12} stat (1 mg folate and 1 mg vitamin B_{12} parenterally).

Drug Dosage

(i) Rapid replenishment scheme for vitamin B_{12}: 1 mg IM/SC cyanocobalamin/day (week 1), 1 mg twice weekly (week 2), 1 mg/week for 4 weeks, and then 1 mg/month for life.

(ii) Alternatively, after rapid replenishment of vitamin B_{12} stores in first month, 2 mg vitamin B_{12} tablets orally daily (1% absorbed daily via passive diffusion).

(iii) Patients with food-vitamin B_{12} malabsorption require minimum 1 mg vitamin B_{12}/day orally.

(iv) Vegetarians/poverty-imposed near-vegetarians: Rapidly replenish stores with oral 2 mg/day vitamin B_{12} for 3 months and then 5–10 μg daily (tablets or vitamin B_{12}-fortified foods) lifelong.

(v) Subclinical vitamin B_{12} deficiency: Course is unpredictable. Two options: either watch-and-wait until symptoms *or* treat preemptively for 6 months with oral vitamin B_{12} 2 mg/day (and reassess); latter approach (my preference) avoids potential for subtle (often clinically unrecognized) cognitive dysfunction.

(vi) Oral folate (folic acid) 1–5 mg/day sufficient replacement.

Prognosis

(i) General response to vitamin B_{12} replacement is dramatic improvement in sense of well-being, alertness, good appetite, and resolution of sore tongue.

(ii) Megaloblastic hematopoiesis reverts to normal within 12 h and resolves by 48 h.

(iii) Hypersegmented polymorphonuclear neutrophils remain in peripheral blood smear ~14 days.

(iv) Reticulocyte count peaks days 5–8.

(v) Complete blood count normalizes by 3 months.

(vi) Most neurologic abnormalities improve in ~90% of patients with documented subacute combined degeneration, and most signs/symptoms <3 months are reversible.

Causes of Incomplete Response

(i) Wrong diagnosis

(ii) Underlying untreated iron/thyroid deficiency

(iii) Infection (parvovirus B19)

(iv) Uremia

(v) Patient on drug that perturbs DNA (antiretroviral/antimetabolites, etc.)

Prophylaxis of Vitamin B_{12} and Folate Deficiency

Refer Tables 6.1 and 6.2

Further Reading

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Sickle Cell Disease and the Hemoglobinopathies

7

Lydia H. Pecker, Carolyn Hoppe, and Jane A. Little

Overview

a. Oxygen is the main fuel for cells in the human body, generating ATP during oxidative metabolis (via the Krebs Cycle and redox reactions). The primary role of hemoglobin is to safely deliver oxygen to cells for these reactions. Hemoglobin (Hb) also has a major role in normal oxidative stress, the cumulative damage, and aging of red blood cells (RBCs), and this can be accelerated in Hb gene disorders. Overall, defects in Hb structure or function may be profound in affecting the health and function of people with these disorders.

   Elemental oxygen and iron are usually maintained within the heme pocket of each of the two alpha- (α-) and two beta- (β-) globin chains that comprise Hb and are not harmful when ferried through the body within an RBC. In the two most common inherited globin gene disorders, sickle cell disease (SCD) and thalassemia, *oxygen delivery is impaired* due respectively to (1) hemolysis with release *of toxic-free iron*, *heme*, and *oxygen* in the plasma and vaso-occlusion as a result of increased red cell adhesion and (2) reduced or absent globin synthesis and lack of RBC production.

   1. SCD is characterized by severe anemia, associated cardiorenal maladaptation, and lifelong exposure to the toxic consequences of hemolysis. The dangerous components of hemolysis are elevated cell-free iron, heme, and oxygen that result in varying degrees of chronic organ damage, vasculopathy, inflammation, and hypercoagulability.
   2. Thalassemia is characterized by severe anemia with impaired oxygen delivery resulting in ineffective extramedullary hematopoiesis (an expanded "erythron" comprised of red cell precursors) and clinically manifests as delayed development, failure to thrive, and often maladaptive cardiovascular compensation in the untreated growing child.

b. Globin gene mutations may be qualitative (*SCD, β-chain mutation*), quantitative (*α- or β-thalassemia*, named for the chain that is deficient), or both (Hb Constant Spring is an α-globin gene mutation, HbE is an adult β-globin gene mutation). As a whole, globin gene mutations are thought to persist because of the protection they afford in the mild or asymptomatic heterozygous state against severe malarial infections.

Qualitative Abnormalities in Hemoglobin

Overview SCD is the most common worldwide qualitative Hb gene disorder and is a β-globin gene defect. SCD is a term that encompasses both homozygous HbSS, known as sickle cell anemia (SCA), and compound heterozygous sickle cell disease. In SCA, comprising HbSS or, less commonly, HbSβ^0, >85% of the total Hb in the RBC is HbS (Fig. 7.1a). In HbSC and HbSβ$^+$ thalassemia, also known as compound heterozygous or sickle variant disorders, each RBC contains Hb derived from a mix of mutant chains; in HbSC, 50% of the Hb in the RBC is HbS and 50% is HbC (Fig. 7.1b), and in HbSβ$^+$ thalassemia, 80% of the Hb in the RBC is HbS, with approximately 20% normal adult Hb (HbA). In the carrier state, sickle cell trait, HbAS, each RBC contains about 60% HbA and 40% HbS, due to differences in affinity by the wild-type and mutant β-chain for the α-chain. Other abnormal, geographically distinct, β-globin gene mutations that are compound heterozygous "partners" with HbS include HbLepore and HbE. These mutations are

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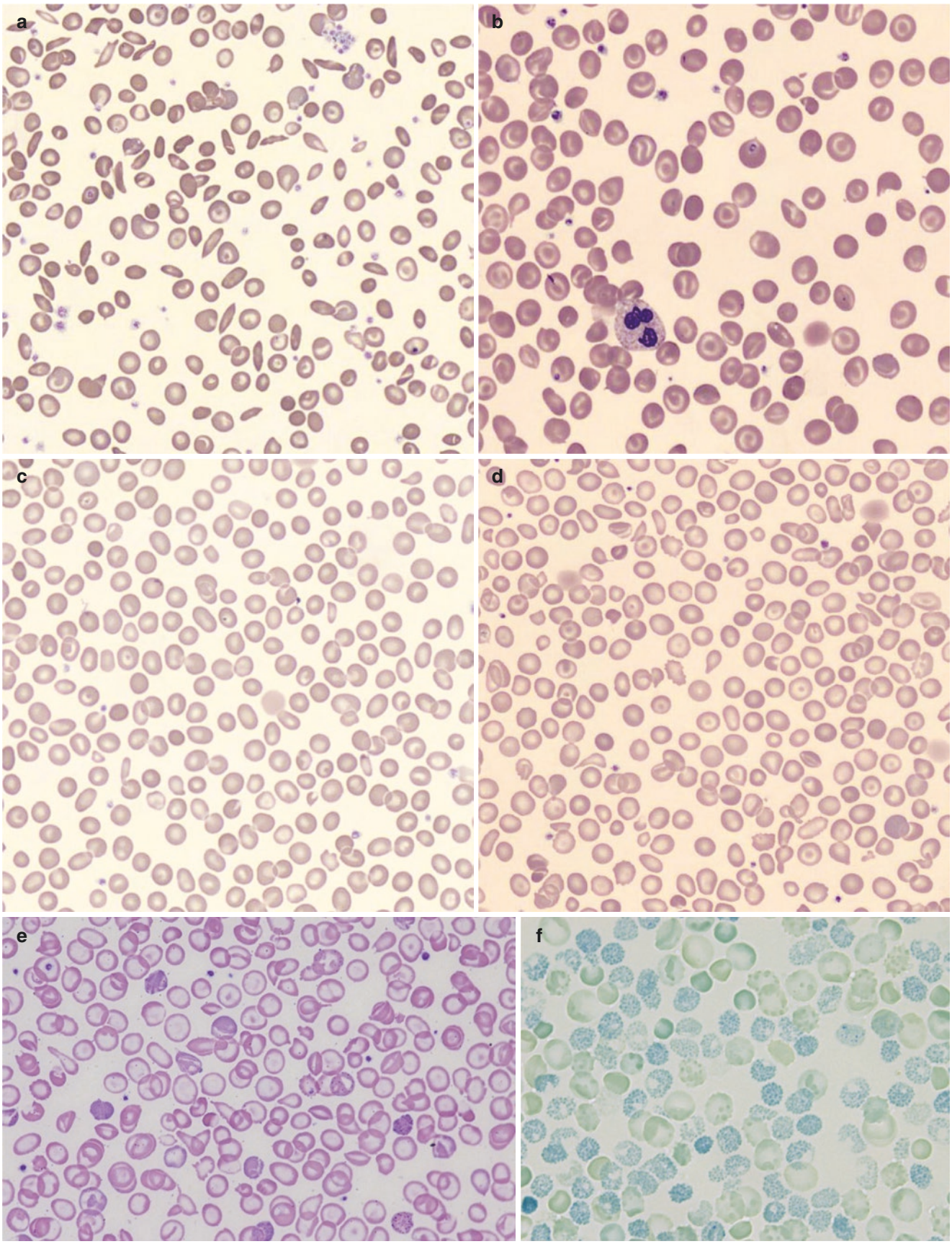

Fig. 7.1 Peripheral blood smears in the hemoglobinopathies. (**a**) Hemoglobin SS disease, (**b**) hemoglobin SC disease, (**c**) α-thalassemia trait, (**d**) hemoglobin H disease/α-thalassemia intermedia, (**e**) HbBart's/homozygous α-thalassemia on Giemesa stain, (**f**) Heinz body prep in HbBart's/homozygous α-thalassemia, and (**g**) β-thalassemia

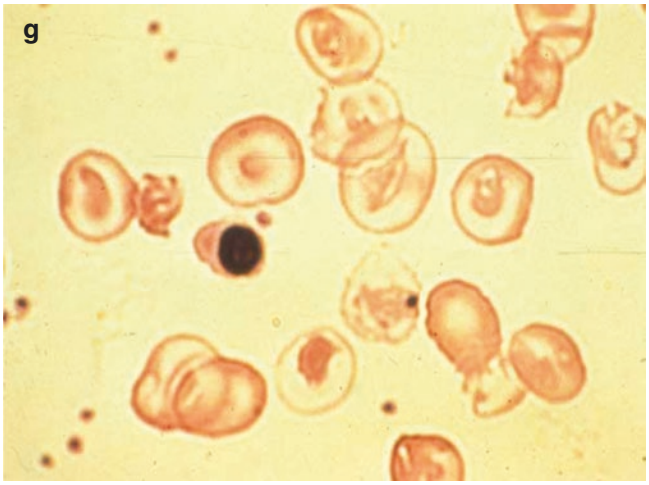

Fig. 7.1 (continued)

Table 7.1 Sickle cell disease subtypes

SCD subtype	HbS, %	HbA, %	HbC, %	Total Hg, g/dL
SCA, HbSS, or HbSβ^0	>85	0	0	HbSS ~8
SCA transfused	15–85	15–85	0	8–10
HbSC	50	0	50	~11
HbSβ$^+$ thalassemia	80	20	0	~11
Sickle cell trait, HbAS	40	60	0	14

common in East Asia. Table 7.1 contains a summary of the four most common sickle cell disease genotypes.

a. Epidemiology: The HbS mutation of the β-chain persists in the malaria belt of sub-Saharan Africa (three independent haplotypes), Saudi Arabia and South Asia (at least one independent haplotype), and in countries of the African diaspora (the USA, the Caribbean, South America, especially Brazil, and the Mediterranean). With time, the antimalarial but less harmful β-chain HbC mutation is predicted to overtake the HbS mutation in geographic regions of Africa where they coexist. Approximately 1.5 million Americans have sickle trait (HbAS) and about 100,000 Americans have SCD, most with homozygous HbSS but up to one-third with HbSC; 1 in 1941 live births is affected by SCD (approximately 1 in 3186 and 1 in 6173 live births, respectively, are affected by HbSS or HbSC), and newborn screening is universal in the USA. In contrast, it is estimated that over 200,000 babies are born with SCD annually in sub-Saharan Africa, a minority of whom undergo newborn screening.
b. Prognosis: An expanding adult SCD population is a relatively recent phenomenon, seen primarily in resource-rich healthcare settings. The median life expectancy for SCD in the USA in the mid-1970s was approximately 15 years of age and is now three to four decades longer. People with compound heterozygous SCD have a longer life expectancy than do people with homozygous SCD, although all patients can be afflicted with sudden life-threatening cardiopulmonary and infectious complications.
c. Pathophysiology:
   i. *Molecular*: The sixth codon in the β-chain is the site of both the sickling HbS (GAG to GTG) and the dehydrating HbC (GAG to AAG) mutations. These point mutations produce abnormal globin proteins. HbS forms unique polymers that damage and distort the RBC membrane. HbC forms intracellular crystals that alter membrane transporter function, resulting in RBC dehydration. In HbSC, this can cause HbS to sickle even when present at only 50% of the total Hb.
      1. Tissue-level hypoxia may significantly increase HbS polymerization and RBC sickling.
   ii. *Physiology*: Sickle polymer formation within an RBC results in two interrelated phenomena, vaso-occlusion and hemolysis. An acquired immunodeficiency also results from splenic damage (below).
      1. *Vaso-occlusion*: Abnormal damaged RBC membranes adhere abnormally to other cells (endothelial, inflammatory, platelet, red cell) and cause a cascade of injury.
         (a) Enhanced cellular adhesion may cause local ischemia and exacerbate hemolysis.
         (b) Acute and chronic pain arise from short-term and long-term effects of vaso-occlusive crises (VOCs). Recurrent VOCs are a major cause of morbidity for most people with SCD.
      2. *Hemolysis and Vasculopathy*: Hemolysis and a shortened RBC half-life cause the release of cell-free Hb (and lactate dehydrogenase, LDH) into the plasma.
         (a) Plasma-free Hb is a reactive oxygen species that also depletes the major vasorelaxant nitric oxide, NO.
         (b) Cell-free heme damages the endothelium and is pro-inflammatory and thrombophillic.
         (c) A shortened RBC half-life imposes significant metabolic demands on the body. Untreated children with homozygous HbSS are small for age and experience delayed sexual maturity.
      3. *Acquired Immunodeficiency of SCD*: Abnormal RBC shape and membrane properties cause early splenic damage and, eventually, "auto-infarction" of the spleen with loss of splenic function in children with homozygous SCA. The spleen is a major site of bacterial opsonization and its damage and eventual loss places people with SCA, more than SCD, especially children, at risk for life-threatening bacterial infections. Mitigating the risk of bacterial sepsis through penicillin prophylaxis and immunizations is one of the major victories attributed to universal newborn screening in the USA.
d. Clinical Presentation and Complications (Summarized in Table 7.2)

Table 7.2 Clinical complications of SCD

Infection	Bacteremia, infections attributed to encapsulated organisms, osteomyelitis (esp. *Salmonella*)
Cerebrovascular	Silent cerebral infarct, acute ischemic stroke, acute hemorrhagic stroke, vasculopathy, neurocognitive impairment, headache, learning disability (lower IQ)
Pulmonary	Acute chest syndrome, pulmonary emboli, pulmonary hypertension
Cardiac	Sudden cardiac death, cardiomegaly
Hepatic/GI	Hepatopathy, acute hepatic sequestration, intrahepatic cholestasis, cholelithiasis, splenic sequestration, hypersplenism,
Musculoskeletal	Avascular necrosis, painful crises
Renal	Hyperfilitration, nephropathy, papillary necrosis, chronic kidney disease
Thrombotic	Pulmonary emboli, venous thromboembolic disease
Genitourinary	For men: priapism, enuresis, diminished sperm counts
	For women: dysmenorrhea
Hematologic	Anemia, leukocytosis, thrombocytosis, microcytosis (when alpha-thalassemia coinherited), macrocytosis (on hydroxyurea or if folate or B12 deficient), thrombocytopenia (with splenic sequestration acutely, or with splenomegaly or side effect of hydroxyurea chronically)
Opthalmalogic	Retinopathy, acute retinal artery occlusion
Other	Painful crises, learning impairment, and growth delay

i. *At Birth*. Non-sickling fetal Hb (HbF) predominates until 6–12 months of age, affording a welcome delay in symptom onset. In regions where SCD is highly prevalent but uniform newborn screening is unavailable (e.g., Nigeria and the Republic of Congo in Africa and central and eastern India), this 6-month window is a lost opportunity for preemptive prevention and treatment of life-threatening infections and other sickle cell-related complications in childhood.

ii. *Infants and Children*. Early causes of mortality in young children with sickle cell disease include:
   1. Life-threatening infections, especially with encapsulated organisms. This risk is ameliorated by penicillin prophylaxis, vaccination for encapsulated organisms, and aggressive hospital-based management of fever through evaluation and empiric administration of a third-generation cephalosporin.
   2. Splenic sequestration (painful splenomegaly with depressed Hb and platelets but preserved reticulocytosis), before the spleen has atrophied. Blood transfusions may be needed to the treat abrupt and severe anemia that may arise from this complication. Parents are taught to palpate their affected child's spleen for enlargement, which prompts urgent medical evaluation.
   3. Aplastic anemia, often virally mediated (especially parvovirus), may result in a profound and life-threatening anemia characterized by reticulocytopenia. Supportive treatment with blood transfusions is often required.
   4. Progressive cerebral vasculopathy and/or ischemic stroke. In the USA and UK, children with homozygous HbS undergo screening for changes in cerebrovascular blood flow via transcranial Doppler examination of the cerebral vasculature. When present, cerebral vasculopathy is managed with chronic red cell transfusions. Transcranial doppler screening and chronic transfusion, when indicated, has contributed to a significant decrease in the burden of stroke in children with SCA. Subtle impairments in cognition may arise from subclinical cerebral vasculopathy that cause silent cerebral infarcts, which are not routinely managed with transfusions, although this is under study.

iii. *Adolescents and Young Adults*. Adolescence is a particularly vulnerable time for chronically ill patients with SCD due to hormonal changes, early manifestations of chronic organ damage from ongoing hemolysis and vaso-occlusion, and a diminution in family, community, and social support during the transition from pediatric to adult care.

iv. *Adulthood*. Adults with SCD, especially SCA, experience complications from cumulative organ damage, notably central nervous system (risk of stroke and intracerebral hemorrhage), cardiac (diastolic dysfunction, arrhythmias), pulmonary (acute chest syndrome, asthma, pulmonary hypertension, pulmonary emboli), gastrointestinal (liver damage from SCD or iron overload), renal (nephropathy, progressive renal failure, acidemia, low erythropoietin, relative hypertension), and musculoskeletal (avascular necrosis). Reproductive health is affected by recurrent priapism (VOC of the penis) that can cumulatively result in impotence in men. In women, pregnancy is managed by high-risk obstetrics. Because of the intermittent but progressive nature of the illness, adults with SCD face barriers to uninterrupted schooling and full employment, with reduced college completion rates and lower incomes compared to race- and age-matched peers.

v. *Compound Heterozygous SCD*, including HbSC and HbSβ^+thalassemia, usually confers a milder clinical phenotype overall and a longer life expectancy. These patients tend to have a higher baseline Hb and a lower hemolytic rate than do patients with SCA. In HbSC (the paradigmatic compound heterozygous state), abnormal RBC dehydration, cellular adhesion, and hyperviscosity contribute to disease pathophysiology. Patients with compound heterozygous SCD may be at

risk for hypersplenism rather than splenic atrophy, and high rates of retinopathy are documented in HbSC. In addition, these patients experience thrombophilic complications, avascular necrosis of the bones, and chronic more than acute pain, which may add significantly to daily symptom burden. However, like patients with SCA, patients with compound heterozygous SCD can be afflicted by VOC and its life-threatening variants, acute chest syndrome and multi-organ failure.

e. Treatment (Summarized in Table 7.3)
   i. *Preventive Health*. Daily PCN and broadened immunizations in youngsters with SCD have significantly decreased disease-related mortality. Supplementation with folic acid, which is depleted from chronic excessive erythropoiesis, is used in some settings and is important in countries where there is no universal folic acid supplementation of processed breads and cereals.
   ii. *Augmentation of Fetal Hemoglobin*. HbF is a potent anti-sickling Hb, while HbA is not (i.e., 20% HbF in homozygous SCD may have a mild clinical phenotype, while 20% HbA in HbSβ$^+$ thalassemia yields a significant clinical phenotype). This explains the milder clinical phenotype seen in patients with hereditary persistence of fetal Hb (HPFH), if HbF is present in all cells (pancellular) at high levels (~30%), as well as the efficacy of hydroxyurea, which increases HbF and dampens inflammation. In SCA, hydroxyurea is considered standard of care for adults with symptomatic disease and is being widely adopted for use in children with SCA even if they have not yet developed symptomatic disease.
   iii. *Blood Transfusions*. RBC transfusions are used intermittently to treat sudden life-threatening complications, such as stroke, acute chest syndrome, and sickle hepatopathy, or an acutely worsened anemia (Hb<5–6 g/dL), especially if an appropriate reticulocyte response is absent. Chronic transfusion regimens are used to preserve cerebrovascular health and may not be routinely available in resource-limited settings. Transfusions are not a routinely used for management of acute pain.
      1. Adverse effects of transfusions include:
         (a) Alloimmunization to transfused RBC antigens, which may limit future transfusions.
         (b) Delayed hemolytic transfusion reactions to transfused RBCs, which may occur 1–2 weeks post-transfusion.
         (c) Iron overload, which is cumulative, may be insidiously life-threatening as liver and cardiac iron deposition contribute to organ failure. Oral chelation has dramatically improved the management of this secondary hemochromatosis.
   iv. *Hematopoietic Stem Cell Transplant, Gene Therapy, and Precision Medicine*. Significant strides have been made in increasing the availability of stem cell transplantation as a cure for SCD, including a recent expansion to haploidentical donors. Remaining barriers include short-term toxicity (infections, graft-versus-host disease) and long-term consequences (reproductive failure) of the preparative regimen and transplant. Mild preperative regimens, which appear to be better tolerated, show up to 50% graft rejection rates. Clinical studies are ongoing, and it is likely that new insights (about graft rejection, anti-sickling globin chains, and the molecular regulation of the fetal β-globin chain) will inform graft manipulation and targeted non-transplant molecular therapies in the future. The decision of who to transplant, and when to transplant them, remains a challenge in SCD, a lifelong progressive disease with significant morbidity but decreasing mortality. In children with homozygous SCD, matched sibling transplants have greater than 90% event-free survival and overall survival. For this reason, HLA typing is increasingly offered to children with homozygous SCD and their potential matched sibling donor.

Table 7.3 Summary of treatments for globin gene disorders

Disorder	Treatment	Comments
Sickle cell anemia	Hydroxyurea	Increase HbF and total hemoglobin
		Alters blood cell indices:
		Increases MCV
		Decreases ANC
		Decreases platelet count
		Decreases WBC
		Skin and nail changes, weight gain
	Chronic red cell transfusion	Risk for iron overload and alloimmunization
	Bone marrow transplant	Infection risk, GVHD or graft rejection, limited donor pool, ?fertility
Sickle cell disease	Chronic red cell transfusion	Risk for iron overload and alloimmunization
Beta-thalassemia	Chronic red cell transfusion	Risk for iron overload and alloimmunization
	Bone marrow transplant	Infection risk, GVHD. ?fertility
	Gene therapy	Risks not fully characterized
Alpha-thalassemia intermedia	Transfusions as needed	Risk for iron overload and alloimmunization
Homozygous alpha-thalassemia	Intrauterine transfusion	Contingent on prenatal diagnosis
	Chronic transfusion	Risk for iron overload and alloimmunization
	Bone marrow transplant	Rare, few cases to date, infection risk, GVHD, ?fertility

?fertility may have effects on fertility and fertility preservation is offered

If a match is identified, a referral to transplant for consideration of curative therapy may be made. The decision to offer transplant to children who lack a matched sibling donor or to adults is more complicated due to more complicated risk-benefit ratios. In this setting, transplant referral depends on the patient's disease complications, available donor sources, and clinical trial availability. Transplant decisions will be informed by evolutions in transplant management, by availability of matched sibling or "alternative" (half-match related (haploidentical) or unrelated (adult or cord blood)) donors, and patient, family, and physician preference.

f. Diagnosis
   i. *Blood Smear*. Review of a blood smear in homozygous HbSS shows abnormal sickled red cells and prominent reticulocytosis (more than is seen in compound heterozygous disease, Fig. 7.1a, b). In HbSC, hyperchromic (high MCHC) red cells and abnormal folded "pita" shaped red cells are evident but with fewer overt sickled cells. HbSβ$^+$thalassemia may show coincident microcytosis and target red cells, although microcytosis can result from coinherited α-thalassemia in any situation. At birth, review of a blood smear may show no abnormalities because of the presence of high levels of the anti-sickling HbF.
   ii. *Screening Tools*. Newborn screening in the USA and UK is accomplished using the dried blood spot heelstick sample collected at birth and then performing either high-performance liquid chromatography (HPLC) or isoelectric focusing for detection of a hemoglobinopathy, with confirmatory testing via complementary methods.
      1. Simple inexpensive solubility assays, e.g., sickledex, can screen in older children and adults but lack sensitivity/specificity, are user dependent, and should not be used for newborn screening.
      2. Confirmatory tests for hemoglobinopathies include biochemical methods, such as electrophoresis, IEF, capillary electrophoresis, or HPLC. Molecular methods are used for definitive diagnosis of thalassemia or hemoglobinopathies that cannot be resolved by biochemical methods alone.

         Among patients who have emigrated from countries without universal newborn screening, clinical complications such as anemia, hypersplenism, and growth delay (thalassemia or SCD), pain and priapism (SCD), or skeletal abnormalities and nontransfusional iron overload (thalassemia) may be the first manifestation of disease and may lead to diagnosis during or after childhood.

Quantitative Abnormalities in Hemoglobin

Introduction Thalassemia (by the sea) is a globin chain imbalance, typically due to point mutations or deletions, respectively, in the β- and α-globin genes. When heterozygous, thalassemias are usually clinically mild and may or may not show associated hypochromia and microcytosis. Worldwide, these disorders are geographically coincident with the malaria parasite; heterozygous inheritance of thalassemia trait offers some protection from severe malarial infections. With population migration, however, geographic limitations on diagnosis no longer strictly pertain.

α-Thalassemia

Overview The α-globin locus on chromosome 16 contains 2 identical adult α-γlobin genes in *cis* (αα/on the same chromosome) or a total of 4 adult globin genes (αα/αα) in erythroid precursors. Most disease conditions arise from the deletion of one or both of the adult α-globin genes per chromosome, with a maximum of four genes affected per cell. The major nondeletional α-globin gene mutation is Hb Constant Spring (Hb^{CS}), which is caused by a point mutation and causes a qualitative α-globin defect. The clinical consequences of α-globin gene deletions are dictated by the degree of inherited α-globin deficiency (summarized in Table 7.4).

Inheritance of one ("silent carrier," -α/αα) or two (α-thalassemia trait, -α/-α or --/αα) α-globin gene deletions does not usually cause clinically significant disease. The loss or dysfunction of three (α-thalassemia intermedia or Hemoglobin H, HbH disease, --/-α) or four (α-thalassemia major or homozygous α-thalassemia, --/--) α-globin genes causes clinically relevant disease (Table 7.4).

An α^0-mutation refers to the lack of expression in both α-globin genes from the referenced chromosome, while an α$^+$-mutation refers to deficient but not absent production from that chromosome. More than 40 α^0-mutations such as $-^{SEA}$, $-^{FIL}$, and $-^{MED}$ are reported. These mutations are responsible for the major morbidity and mortality related to α-thalassemia when they are coinheritied with an α$^+$−thalassemia (α-thalassemia intermedia/HbH disease) or a second α^0−thalasemia trait (homozygous α- thalassemia/Hb Barts/hydrops fetalis).

(a) *Epidemiology*: α-globin gene mutations and deletions have a carrier rate >1% in tropical and subtropical regions, such as sub-Saharan Africa and Southeast Asia. Up to one-third of African Americans carry a single α-globin gene mutation (silent), and 1 in 50 carry a two-allele

Table 7.4 α-Thalassemia

The α-thalassemias			
Number of deletions or mutations	Syndrome	Genotype	Clinical manifestations
1	Silent carrier	(-α/αα)	Normal or mild decrease of MCV and Hb; occurs in 1 in 3 African Americans
2	α-thalassemia trait	(-α/-α) or (--/αα)	Mild anemia, decreased intracellular Hb and MCV. RBC count is often increased and may be mistaken for iron-deficiency anemia
3	HbH/α-thalassemia intermedia	(--/-α)	Accumulation of abnormal β-tetramers (HbH) leads to hemolytic anemia with a variable degree of anemia. Some patients develop splenomegaly, non-transfusion iron overload, extramedullary hematopoiesis, and sometimes require transfusions
4	Hb Bart's/ hydrops fetalis/ homozygous α-thalassemia	(--/--)	In utero, abnormal γ-tetramers (Hb Bart's) fill α-globin-deficient red cells but are functionally inadequate, leading to hydrops fetalis and, often, 2nd or 3rd trimester fetal demise. This is associated with maternal morbidity and mortality. Prenatal diagnosis, intrauterine transfusion, intensive care, and hematopoietic stem cell transplant are changing prognosis for infants and mothers in resource-rich settings

α-globin gene mutation (trait). Among people of African descent, a single α-globin gene deletion on each chromosome (-α/-α, in "trans" formation) is the most common form of α-thalassemia trait. Southeast Asian variants resulting in α-thalassemia trait are typically inherited in "cis" formation: the two mutated or deleted α-globin alleles are on the same chromosome (--/αα, i.e. $--^{SEA}$). These mutations are carried by as many as 5% of people from Southeast Asia.

(b) *Prognosis*: The disease burden associated with α-thalassemia relates to inheritance patterns. Inheritance of one (silent) or two (trait) gene deletions is clinically benign, whereas a three-gene deletion, HbH disease/α-thalassemia intermedia is associated with chronic complications, which may include intermittent hemolytic episodes requiring transfusion, hepatosplenomegaly, extramedullary erythropoiesis, non-transfusional iron overload, and diminished life expectancy. Homozygous α^0−thalassemia is almost universally fatal, although prenatal diagnostics, intrauterine transfusion, chronic transfusions, and even hematopoietic stem cell transplant are changing the epidemiology of this condition.

Pathophysiology Unlike β-globin gene switching, which occurs over 6–12 months of postnatal life, α-globin gene switching occurs early in prenatal development. Fetal development may be dramatically affected by significant α-globin deficiency. In α-thalassemia, excessive β-type globin chains homodimerize; detection of these may aid in diagnosis. In more severe α-thalassemia, homodimerization of excess fetal β-type globin chains (γ−globin) in the fetus forms tetramers referred to as Hb Bart's ($Hb\gamma_4$). After birth, excess adult β-chains form HbH ($Hb\beta_4$). Hb Bart's and HbH are unstable and precipitate within the cell, producing insoluble inclusion bodies that damage the RBC membrane. These tetramers are dysfunctional, cannot deliver oxygen to tissues, and result in a chronic hemolytic anemia and a shortened red cell half-life.

i. *Clinical Presentation*:
   1. *1-deletion. Silent* carriers do not manifest adverse hematologic or clinical outcomes.
   2. *2-deletion*. α-Thalassemia *trait* shows abnormal red cell indices including mild normochromic or hypochromic anemia with severe microcytosis (MCV 55 – 65 fL) and typically an increased red cell count, which may help distinguish this from iron-deficiency anemia.
   3. *3-deletion*. *HbH disease* (α-thalassemia intermedia) is suspected in infants whose newborn screen reveals elevated levels of Hb Bart's (γ_4). Blood smears show anisocytosis, polychromasia, and increased red cell distribution width (RDW, Fig. 7.1c).
   4. *4-deletion. Hydrops fetalis* (homozygous α-thalassemia) is usually incompatible with life. Suspicion of an affected fetus is heightened among women of Southeast Asian descent with a history of third trimester miscarriage. Hydrops fetalis results in significant maternal morbidity, with mortality rates reportedly as high as 50%. Affected infants who do

not receive intrauterine transfusions are born prematurely in the third trimester when oxygen demands outpace the capacity of the fetus' defective Hb. These infants usually have severe hydrops fetalis and require aggressive resuscitation. Neurocognitive deficits, genitourinary anomalies, and transfusional iron overload are among the complications of surviving children with homozygous α-thalassemia. Preterm delivery, poly- and oligohydramnios, severe preeclampsia, placental abruption, and postpartum hemorrhage are described and may not always be altered by interventions that treat the fetus, such as intrauterine transfusions.

a. *Treatment*: Interventions for the α-thalassemias vary based on the number of inherited mutations (see also Table 7.3).
   i. *1-gene* (silent carrier) and *2-gene* (trait) deletions in α-thalassemia require no therapy. Trait may be suspected in patients with mild anemia, microcytosis, and increased RDW that a trial of oral iron therapy will not fully resolve. Gene analysis, Hb electrophoresis, iron studies, and blood smear review help distinguish α-thalassemia trait, iron deficiency, and β-thalassemia trait (Fig.7.1c–f), although these are not mutually exclusive diagnoses. Preconception counseling is especially important for patients from regions at high risk for α-thalassemia.
   ii. *3-gene* loss (HbH disease/α-thalassemia intermedia) is treated based on anemic symptoms. Supplemental folic acid is often provided. Some, but not all, patients require intermittent or chronic transfusions. Iron overload can result from signaling from the stressed erythron and can be occult, since it may not arise primarily from transfusions.
   iii. *4-gene* deletion (Hb Bart's/hydrops fetalis/homozygous α-thalassemia) is typically life-threatening during fetal life/early infancy. Patients who survive are treated with lifelong chronic transfusions or curative hematopoietic stem cell transplant. Because most infants affected by this mutation are born prematurely in resource-limited settings, the majority of affected fetuses and infants do not survive. New evidence guides management in resource-rich settings. When diagnosed prenatally, Catastrophic perinatal complications may be mitigated by intrauterine transfusions for the fetus, and chronic transfusion therapy can then be initiated after birth. Infant and maternal mortality is further reduced where perinatal critical care and chronic care resources are available.

b. *Diagnosis*
   i. *Silent carrier/α-thalassemia trait* are diagnosed through genetic analyses only.
   ii. HbH disease (α-thalassemia intermedia) is diagnosed when newborn screening reveals Hb Bart's ($Hb\gamma_4$). Heinz body "preps" (such as staining with methylene blue, Fig. 7.1f) will demonstrate red cell inclusions comprised of unstable denatured γ- or β-globin tetramers, depending on whether the developmental β-globin switch has occurred. Molecular testing provides definitive genetic diagnosis.
   iii. *Hydrops fetalis* (homozygous α-thalassemia) is diagnosed pre- or postnatally:
      1. Prenatal genetic diagnosis may be made with polymerase chain reaction techniques, and should be performed in instances where parental heritage (e.g., Southeast Asia) makes this diagnosis likely. Amniocentisis, chorionic villus sampling, or cordocentisis may eventually be supplanted by diagnostic testing of cell-free fetal DNA in the maternal circulation as this technology becomes more widely available.
      2. Ultrasound of the brain and heart is used, especially in low-resource settings where the incidence of α-thalassemia is high. Cardiac:thoracic diameter ratio, systolic velocity of the middle cerebral artery, and placental thickness are among the best studied ultrasonic measures to predict severe fetal anemia.
      3. Hydrops fetalis may be the presenting symptom of α-thalassemia and should be suspected in hydropic infants, especially if their parents are of Southeast Asian descent. Many mothers will have a history of late second and early third trimester miscarriage without a known diagnosis of a hemoglobinopathy.
      4. The presence of hemoglobin Bart's ($Hb\gamma_4$) on newborn screen and, later, HbH ($Hb\beta_4$) supports the diagnosis of α-thalassemia. A supravital stain such as methylene blue reveals Heinz bodies, which are clumps of γ- or β-globin tetramers (Hb Bart's and HbH, respectively) in red cells. Additional molecular testing may be necessary to distinguish HbH disease from α-thalassemia trait in the newborn.
   iv. Hemoglobin constant spring (Hb^{CS}) is a nondeletional α-globin gene mutation common in Southeast Asia. Inheritance of this mutation and one additional α-globin gene mutation leads to a normocytic anemia that is more severe than would otherwise be expected in a person lacking two α-globin genes (trait).

v. In patients in whom α-globin gene mutations are suspected, but not confirmed by PCR, whole α-globin gene sequencing may be necessary.

β-Thalassemia

Overview β-thalassemia can arise from a number of point mutations (less commonly, deletions) in the β-chain, its enhancer, promoter, or introns. This results in a "rheostat" effect, ranging from no β-chain production (β^0) to modestly limited β-chain production (β^+) from the affected chromosome. β-thalassemias are, therefore, clinically defined based on severity of disease, including thalassemia major (no adult β-chain production from either chromosome, total Hgb <5–6 g/dL, usually transfusion-dependent), thalassemia minor (asymptomatic with small or pale red cells, usually not treated), and thalassemia intermedia (depressed Hb, around 8–10 g/dL, possible enlarged spleen, possible intrinsic iron overload). However, these are clinical diagnoses and thalassemia intermedia may transition to thalassemia major (transfusion-dependent) as anemia-tolerant children age into adolescence and adulthood. The genetic cause, i.e., the specific mutation causing the decreased Hb production in β-thalassemia varies regionally.

(a) *Epidemiology*. β-thalassemia has a worldwide distribution and is clinically evident in sub-Saharan Africa, the Mediterranean, India, and Asia, with significant populations found in the North and South American diaspora from these regions.

(b) *Prognosis*. Untreated, thalassemia major is usually fatal during early childhood. HPFH, in which γ−globin chains functionally substitute for the absent adult β-chain, may appear identical to thalassemia major at birth but can be distinguished through molecular testing. Children with β-thalassemia major are afflicted by severe anemia, hepatosplenomegaly, malnutrition, and developmental delay. Thalassemia intermedia has a less predictable clinical presentation and phenotype but at its most debilitating can manifest significant extramedullary erythropoiesis and iron overload, even without severe anemia or transfusion requirements. Thalassemia minor (i.e., β-thalassemia trait or carrier) is clinically silent but may show mild anemia and iron replete microcytosis.

(c) *Pathophysiology*. The α- and β-globin gene clusters do not directly regulate each other, and there is little evidence for feedback. That is, impaired β-chain production is not recognized by the α-gene cluster, resulting in unrestrained α-chain production, despite β-chain deficiency, and resultant globin chain imbalance. The unbound α-chain is particularly noxious, causing significant oxidative damage to the red cell membrane in β-thalassemia. In thalassemia, red cell precursors proliferate exuberantly but differentiate poorly, resulting in increased numbers of small and abnormal red cells. Ineffective erythropoiesis also results in the release of hormones, such as Eryfe from the erythron, which stimulate iron absorption from the gut despite iron repletion in the patient, potentially resulting in significant iron overload in patients with non-transfusion-dependent thalassemia intermedia.

(d) *Clinical Presentation*. β-thalassemia rarely presents at birth, due to the prominent perinatal role of HbF. However, once the switch to adult β-globin chain production is complete, at 6–12 months, progressive anemia, failure to thrive, and hepatosplenomegaly will develop in thalassemia major. Thalassemia intermedia is less prominent and may present during prenatal screening in young women, masquerading as iron-deficiency anemia. These patients may be at risk for iatrogenic iron overload, following empiric treatment of microcytosis with iron. Hypersplenism is common in thalassemia intermedia. Evidence for iron overload should be sought.

(e) *Treatment*. Red cell transfusion, chelation, and early hematopoietic stem cell transplantation are the mainstays of management in thalassemia major. Iron overload, resulting from lifelong exposure to lifesaving blood transfusions, affects the heart and liver and results in death in adolescence unless managed aggressively.

(f) Thalassemia intermedia usually does not require transfusions but may need observation for evidence of hypersplenism and symptomatic extramedullary erythropoiesis. Notably, patients with thalassemia develop significany hypercoagulability following splenectomy (summarized in Table 7.3).

(g) *Diagnosis*

1. Review of a blood smear from a patient with β-thalassemia may show a combination of hypochromia (pale), microcytosis (small, low MCV), and abnormal ("targets") or nucleated red cells (Fig. 7.1g)
2. The β-globin gene cluster includes a weakly transcribed second adult gene, delta (δ), and fetal gene, gamma (γ). Under some circumstances, these gene products will be increased in patients with β-thalassemia and will be detectable as HbA_2 ($\alpha_2\delta_2$) and HbF ($\alpha_2\gamma_2$) on Hb electrophoresis or high-performance liquid chromatography (HPLC).

3. Since Hb analyses are qualitative rather than quantitative, detection of a decreased level of HbA alone may not itself reveal the underlying cause. Unless HbF or HbA_2 are elevated, globin gene sequencing may be necessary to unravel the precise mutation(s) underlying an observed iron replete microcytic hypochromic anemia.

Tests for alpha-/beta-thalassemia: In subjects in whom ethnicity and blood smears and indices suggest globin gene abnormalities, but for whom preliminary screens (electrophoresis, HPLC) are nondiagnostic, full molecular sequencing of at least the α- and β-loci may be necessary for diagnosis.

Suggested Reading

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Congenital Hemolytic Anemias

8

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Introduction

Most congenital hemolytic anemias result from intrinsic defects of the red blood cells (RBCs) that by various mechanisms render the RBC more susceptible to premature destruction. These intrinsic defects can be categorized into membrane defects, enzymatic defects, and hemoglobinopathies. Other rarer congenital hemolytic anemias result from dysregulated hematopoiesis, disorders of complement activation such as those responsible for atypical hemolytic uremic syndrome, and abnormal von Willebrand multimer processing. The clinical presentation ranges from asymptomatic to chronic compensated hemolysis to transfusion-dependent anemia or acute hemolytic episodes.

General Diagnostic Approach to Congenital Hemolytic Anemias

Careful clinical and family history including the inheritance pattern, physical examination, basic laboratory studies, and, in many cases, specialized laboratory studies are required to define the underlying disease process. The history should explore the chronicity of the problem, ethnic and racial background, family history and possible mode of inheritance, underlying or associated medical conditions, and possible precipitating events such as medications. In those defects that cause chronic hemolysis, complications of chronic hemolytic states, including gallstones, transient aplastic anemia crises (due to parvovirus infection), folate deficiency, extramedullary hematopoiesis, propensity to hemochromatosis, and, infrequently, skin ulcers, may manifest. Splenomegaly may be associated with some but not all disorders.

Laboratory Studies

Laboratory blood studies suggesting hemolysis may be abnormal including increased reticulocyte proportion (or absolute reticulocyte count), increased unconjugated (indirect) bilirubin, increased lactate dehydrogenase, and decreased haptoglobin. In severe intravascular hemolysis, hemoglobinuria and hemosiderinuria may also occur.

Laboratory studies may be normal between episodes in defects that cause only intermittent acute hemolysis as is pathognomonic of G6PD deficiency.

Iron overload is assessed by serum ferritin and transferrin saturation and is further quantified by magnetic resonance imaging studies of the liver and heart. Propensity to iron overload in hemolysis is mediated by downregulation of hepcidin by a hormone erythroferrone, produced by erythropoietin-stimulated hyperproliferative erythroid progenitors, discussed elsewhere in this book (Chap. 2).

Peripheral smear: Different morphological forms of RBCs (Table 8.1) can be seen.

Red Blood Cell Membrane Disorders (See Fig. 8.1)

Hereditary Spherocytosis

Overview Hereditary spherocytosis (HS) is a common congenital hemolytic anemia, particularly in individuals of Northern European descent. The inheritance pattern is autosomal dominant in 75% of cases and autosomal recessive or sporadic (de novo mutations) in the remaining 25%.

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H. M. Lazarus, A. H. Schmaier (eds.), *Concise Guide to Hematology*, https://doi.org/10.1007/978-3-319-97873-4_8

Table 8.1 RBC morphology associated with hemolytic anemias

Morphology	Description	Cause	Disease states
Spherocytes	Spherical cells with dense hemoglobin and absent central pallor	Loss of membrane	Hereditary spherocytosis, autoimmune hemolytic anemia
Schistocytes	Distorted, fragmented cells	Traumatic disruption of membrane	Microangiopathic hemolytic anemia (TTP, HUS)
Sickle cells	Sickle-shaped, tapered at both ends	Polymerization of hemoglobin S	Sickle cell disease
Elliptocytes	Elliptical cell	Abnormal cytoskeletal proteins	Hereditary elliptocytosis
Normal			PK deficiency
Intracellular inclusion:	Heinz bodies	Precipitated Hb	G6PD deficiency, glutathione pathway mutations
	Basophilic stippling	Aberrant ribosomal RNA	P5N deficiency

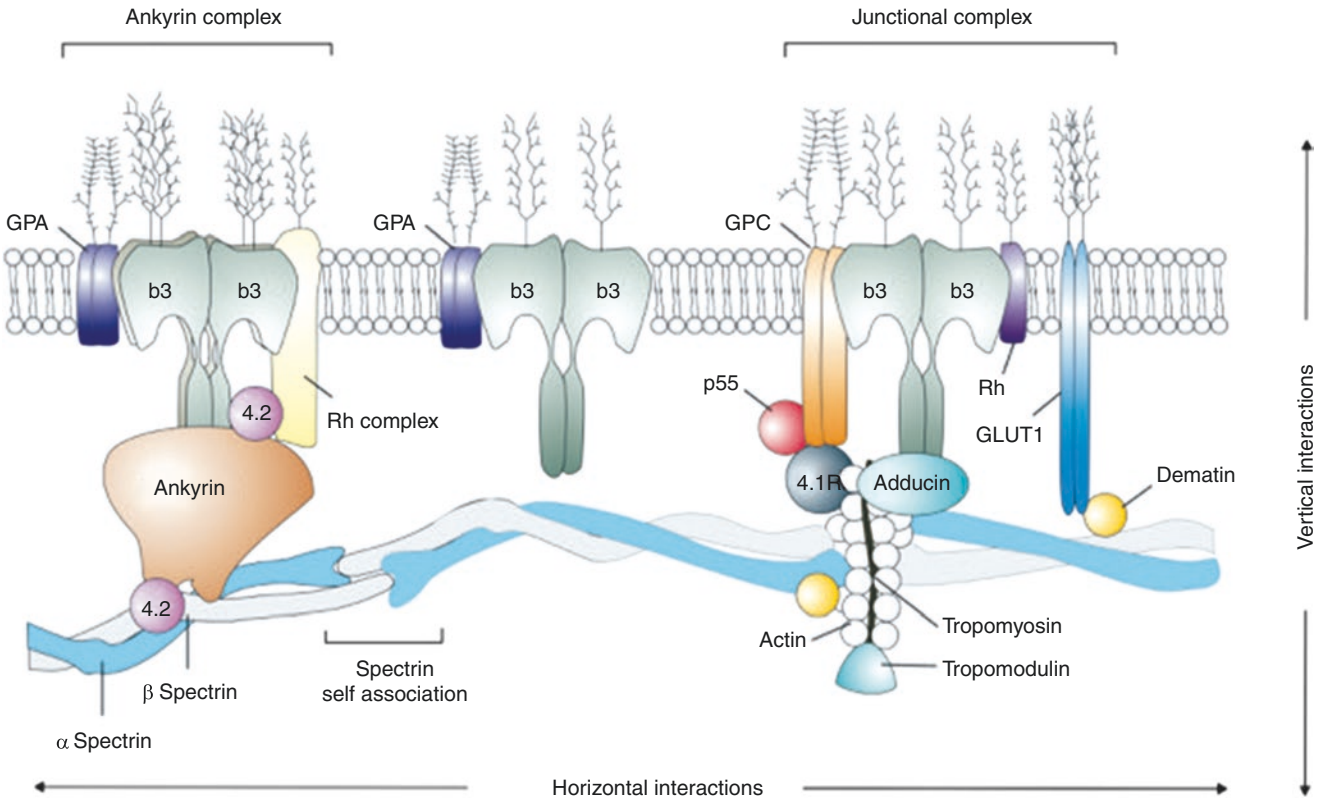

Fig. 8.1 Schematic model of the human erythrocyte membrane. The molecular assembly of the major proteins is indicated. Vertical interactions are perpendicular to the plane of the membrane and are represented by the ankyrin and junctional protein complexes that connect the membrane spectrin skeleton to the integral proteins embedded in the lipid bilayer. Horizontal interactions occur parallel to the plane of the membrane and involve spectrin tetramers and protein 4.1R. The proteins and lipids are not drawn to scale. b3, band 3; GPA/GPC, glycophorin A/C; GLUT-1, glucose transporter-1. (Used with permission from Kaushansky, Williams Hematology, 9th Edition, McGraw Hill, 2015)

Pathophysiology HS is due to mutations of genes encoding RBC structural proteins, most commonly ankyrin (but also band 3, β-spectrin, α-spectrin, and proteins 4.1 and 4.2), that mediate *vertical* interactions of the RBC cytoskeleton with the lipid bilayer. Alpha-spectrin mutations are commonly responsible for autosomal recessive form of HS. Assembly of spectrin onto the cytoskeleton is impaired due to alterations in these proteins, resulting in spectrin deficiency, which correlates with the severity of disease. Spherocytes form as RBC membrane surface is lost through microvesiculation due to the weakened bilayer-cytoskeleton association. The rigidity of spherocytes leads to trapping in the spleen, where the RBCs lose additional membrane and become microspherocytes. Ultimately, after repeated trips through the splenic circulation, microspherocytes are trapped and destroyed. The proportion of spherocytes decreases after

splenectomy but persists. The hemolysis may be completely (no anemia) or partially compensated by an increase in the production of new RBCs by the bone marrow.

Clinical Presentation The clinical manifestations are variable, reflecting the heterogeneity of the molecular defects that underlie the disease. These range from no anemia to severe transfusion-dependent anemia or episodes of acute anemia due either to aplastic anemia (parvovirus-associated) or to acute hemolysis that can be triggered by infection. Affected members of a given kindred will often, but not always, exhibit a similar pattern. Mild to moderate splenomegaly may be present as well as gallstones and other complications of chronic hemolysis. Mild HS may not become clinically apparent until adulthood.

Laboratory Evaluation Anemia and reticulocytosis are usually present, and spherocytes are always seen on the peripheral blood smear.

The presence of spherocytes is not *specific* for HS as these are also seen in autoimmune hemolysis and rare conditions such as recluse spider bite and clostridium sepsis. Spherocytes are objectively detected by the *osmotic fragility test*, but this test is not specific for HS and is laborious. This test measures the in vitro lysis of RBCs suspended in solutions of progressively decreasing osmolarity. Spherocytes, having lower surface to volume ratio, are less able to withstand hypotonicity and rupture in those hypotonic solutions that normal RBCs can withstand. Thus, spherocytes formed by any mechanism (e.g., HS, autoimmune hemolytic anemia, etc.) will yield a positive result. In contrast to spherocytosis from immune hemolytic anemia, the direct antiglobulin test is negative in HS.

The *eosin-5-maleimide (EMA) binding test* is increasingly used as clinical laboratory assay for HS. It has a higher sensitivity and specificity than the osmotic fragility test. It is rapidly performed on a small volume of RBCs and is standardized and thus reproducible. EMA binds to the RBC band 3 protein, allowing its quantitation by flow cytometry; a reduced level of band 3 fluorescence correlates with spherocytosis in most, but not all, cases of HS.

Treatment Most RBC destruction occurs in the spleen, so splenectomy usually ameliorates anemia and always decreases the rate of hemolysis. Splenectomy is recommended only for patients with symptomatic anemia. After splenectomy, the rate of hemolysis and the degree of spherocytosis are decreased, but some spherocytes persist and are readily visible present on the peripheral blood smear; however, RBC survival is markedly improved. Splenectomized patients are at risk for serious infections and should receive appropriate antimicrobial vaccinations; this risk is greatly increased in infants, and thus, whenever possible, splenectomy should be postponed until after 3 years of age.

Hereditary Elliptocytosis and Hereditary Pyropoikilocytosis

Overview Hereditary elliptocytosis (HE) and hereditary pyropoikilocytosis (HPP) are related disorders with a wide spectrum of clinical phenotypes. HE is autosomal dominantly inherited and results from defects in RBC structural proteins that mediate *horizontal* interactions in the RBC cytoskeleton. Most cases are due to a molecular defect, most commonly in either the α- or β-spectrin gene, that prevents normal polymerization of spectrin molecules. Mutations of genes encoding protein 4.1 and glycophorin C can also generate the elliptocytic phenotype. Combined heterozygosity for a missense α-spectrin gene mutation combined with an α-spectrin mutation that decreases transcript of an affected allele (α-spectrin LELY) is associated with HPP, which has distinct easily recognizable morphology.

Clinical Presentation The clinical features and natural history of HE/HPP may be similar to those of HS, including marked variability in the severity of the hemolysis from most commonly none (unlike HS) to mild compensated hemolysis (HE), depending on the specific mutation, to the severe hemolytic phenotype of HPP.

Laboratory Evaluation Elliptocytes are seen on the peripheral blood smear. The elliptical shape results from deformation of the RBC as it traverses the microcirculation, with a failure to revert to the normal biconcave disk morphology. Marked anisopoikilocytosis with elliptocytes, microspherocytes, and bizarrely shaped RBCs are characteristically seen in HPP.

Treatment No therapy is required for most individuals. In HPP, splenectomy only slightly ameliorates the hemolysis. As in any severe chronic hemolytic condition, parvovirus-caused aplastic crisis should be considered with any increase of anemia and appropriately treated.

Hemolytic Enzyme Disorders

Overview Enzyme abnormalities causing hemolytic anemia are involved in glucose metabolism, glutathione metabolism, and the nucleotide-scavenging pathway (see Fig. 8.2). RBCs are uniquely susceptible to enzyme deficiencies because they are unable to synthesize additional protein after being released into the circulation. Only three enzyme defects, glucose-6-phosphate dehydrogenase (G6PD) deficiency, pyruvate kinase (PK) deficiency, and pyrimidine 5′ nucleotidase (P5N) deficiency, occur with any significant frequency.

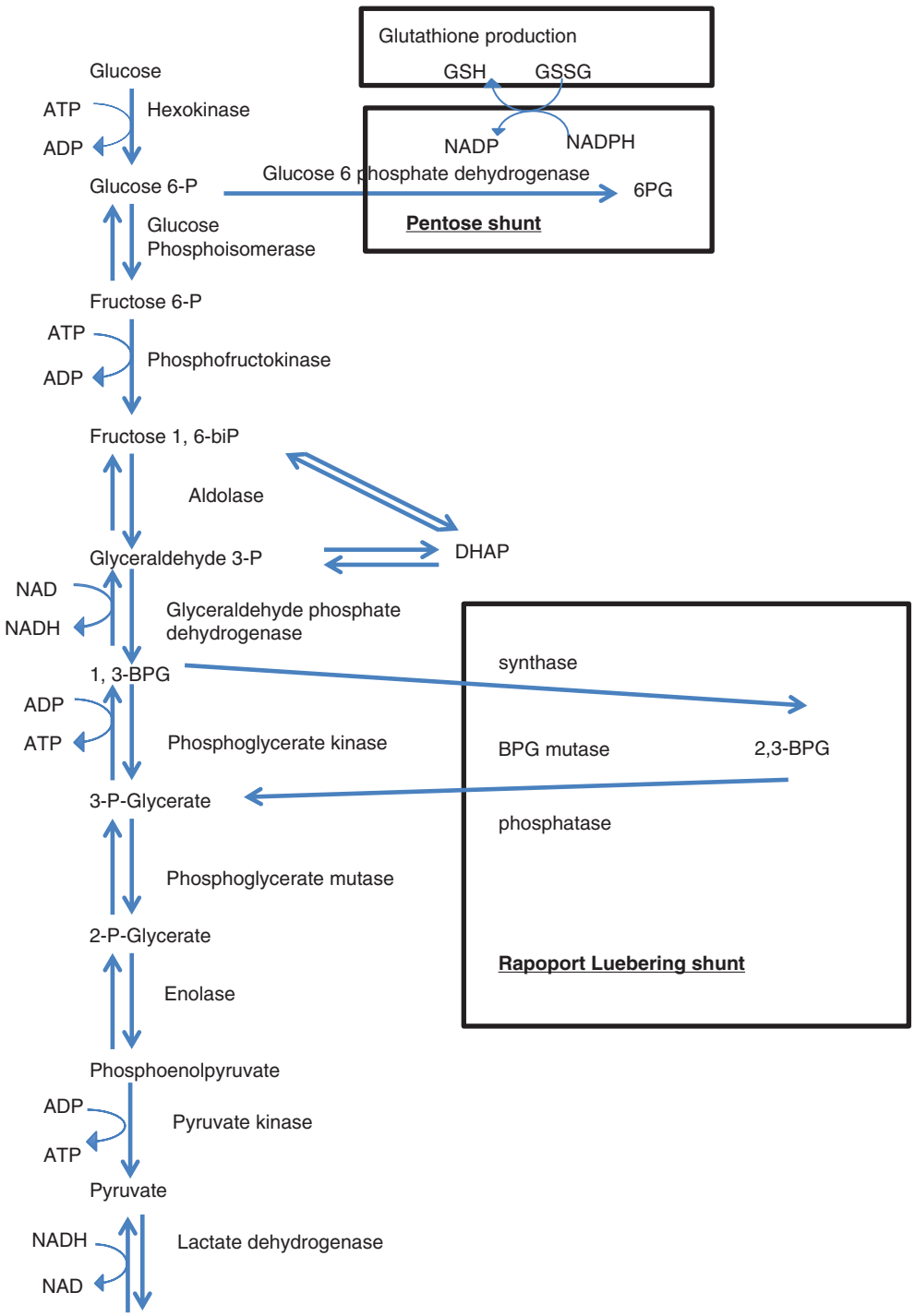

Fig. 8.2 Overview of glucose metabolism of RBCs: glycolysis catabolizes glucose to pyruvate and lactate, pentose shunt oxidizes glucose-6-phosphate, reducing NADP to reduced NADP (NADPH), and Rapoport-Luebering shunt leads to formation of 2,3 BPG. p phosphate, ADP adenosine diphosphate, ATP adenosine triphosphate, NAD nicotinamide adenine dinucleotide, NADH reduced form of NAD, DHAP dihydroxyacetone P, BPG biphosphoglycerate, 6PG 6-phosphogluconate, GSH reduced glutathione, GSSG oxidized glutathione, NADP nicotinamide adenine dinucleotide phosphate, NADPH reduced NADP

Glucose-6-Phosphase Dehydrogenase Deficiency

Epidemiology G6PD deficiency is the most common human enzymopathy, affecting as much as 10% of the world's population (more than 400 million people). The geographic distribution of G6PD deficiency coincides with the distribution of tropical malaria; however, most affected subjects never experience significant anemia or detectable hemolysis.

G6PD deficiency follows a typical X-linked recessive inheritance pattern, with males commonly affected. Heterozygous females have a mosaic distribution of cells, with variable proportions of deficient and normal cells, and the deficient cells are prone to the same degree of hemolysis as those of similarly affected hemizygous males. Thus, the risk of hemolysis in heterozygous females ranges from none to the same as hemizygous males.

Pathophysiology G6PD, a pivotal enzyme in the hexose monophosphate shunt, mediates generation of reduced nicotinamide adenine dinucleotide phosphate (NADPH), which in turn, reduces glutathione. Reduced glutathione is a major free radical (oxidants) scavenger in the RBC. Thus, increased oxidant exposure may result in acute hemolysis. Precipitates of oxidized, denatured hemoglobin (Hb), i.e., Heinz bodies, cause membrane damage and can be visualized on staining with crystal violet. RBCs containing Heinz bodies are recognized and preferentially destroyed by the spleen. On the other hand, so-called "bite" erythrocytes, while alleged to be a specific morphologic finding, have never been proven to be pathognomonic for G6PD deficiency. When oxidant exposure is massive, an acute intravascular hemolysis event known as *blackwater fever* occurs, especially in G6PD-deficient malaria-infected infants.

Classically, oxidant stress is induced by exposure to a variety of chemicals or medications. Ingestion of fava beans may be the commonest hemolysis precipitant in particular in Mediterranean region where fava beans are staple. Acute viral or bacterial infection may also precipitate acute hemolysis.

Variants and Clinical Features of G6PD Deficiency More than 400 variants of G6PD have been described, although only few are endemic. These include the African variant G6PD A-, the Mediterranean variant (notably in Arabs, Southern Europeans, Iranians, Turks, and Sephardic Jews), and several Southern Asian variants common in Southern China, Southeast Asia, and India. Individuals with these variants develop *acute transient hemolytic anemia* only after exposure to oxidants but are asymptomatic and not anemic under normal conditions. Only extremely rare G6PD-deficient variants are associated with chronic hemolytic anemia. Splenomegaly is *not* a feature of G6PD deficiency. Neonatal jaundice, which may result in kernicterus, is a common consequence of G6PD deficiency.

Laboratory Evaluation G6PD activity in RBCs that survive an acute hemolytic attack may be normal in some G6PD-deficient variants, so measurement of G6PD activity immediately after a hemolytic attack or in heterozygous G6PD-deficient females may not correctly identify G6PD deficiency. However, in both these instances, DNA-based tests will result in an accurate diagnosis.

Pyruvate Kinase Deficiency

PK deficiency is by far the commonest RBC enzymopathy causing chronic hemolysis, since G6PD-deficient variants causing chronic hemolysis are extremely rare. The phenotype of PK deficiency is of variable severity with no consistent or specific RBC morphological abnormalities. It is an autosomal recessively inherited defect, and in most sporadic cases, the individuals are compound heterozygotes. In PK deficiency due to consanguinity or in certain population isolates, the affected individuals are homozygous for the same PK mutation.

The mechanism of hemolysis in PK deficiency is not understood, but decreased ATP in RBCs may play a role. However, the block in glycolysis caused by PK deficiency results in increased 2,3-diphophoglycerate levels and a right shift in the oxyhemoglobin dissociation curve resulting in decreased affinity of Hb for oxygen that facilitates oxygen delivery at the tissue level, which permits better tolerance of the anemia in PK deficiency.

The diagnosis of PK deficiency is made in specialized labs by direct measurement of enzyme activity in RBCs. Molecular testing for diagnosis of PK deficiency is increasingly available and is mandatory for prenatal diagnosis.

Severe disease may require RBC transfusions throughout infancy and into adulthood. Splenectomy ameliorates the severity of hemolysis, although, paradoxically, the reticulocyte count increases after splenectomy. Iron chelation is sometimes required. Two oral small molecules that increase the activity of some but not all PK mutants are in clinical trials.

Pyrimidine 5′ Nucleotidase Deficiency

Pyrimidine 5′ nucleotidase (P5N) participates in RNA degradation in reticulocytes. The accumulation of pyrimidines in RBCs is presumed to be toxic and a cause of hemolysis. P5N deficiency is inherited in an autosomal recessive fashion, and it is the only congenital hemolytic anemia due to an RBC enzyme deficiency that has a specific, consistent morphological abnormality—basophilic stippling. Lead is a powerful inhibitor of P5N, and lead toxicity can generate the same phenotype as the congenital P5N mutations.

Other Hemolytic RBC Enzyme Deficiencies

Deficiencies of other enzymes involved in glutathione metabolism besides G6PD can cause either chronic or acute intermittent hemolysis as well as Heinz bodies, which may

be seen continuously or only during an acute hemolytic episode. These defects are exceedingly rare and include glutamylcysteine synthetase and glutathione synthetase and glutathione reductase.

Other rare RBC enzyme defects that are associated with hemolysis and also other systemic disorders such glycogen storage disorders, myopathies, and retardation include deficiencies of phosphofructose kinase, aldolase, phosphoglycerokinase, triose-phosphate isomerase, and transaldolase.

Hemolytic Hemoglobinopathies

Overview Some globin gene mutations may be associated with hemolysis. These abnormalities are further subdivided into quantitative defects of Hb synthesis (e.g., the thalassemias) or qualitative defects of Hb (e.g., unstable Hbs and sickle cell anemia) (see Chap. 7).

Thalassemias

Overview The thalassemias are characterized by a globin chain imbalance. Normally, the production of α- and β-globin chains within an RBC is identical. Mutations that partially or completely inactivate production of either α- or β-globin chains can result in α- and β-thalassemia, respectively. Thalassemia trait/minor phenotypes are present in heterozygous states and usually have mild hypochromic and microcytic RBCs, but hemolysis does not occur (see Chap. 7).

Alpha-Thalassemias

These most commonly occur due to deletion of one or more of the four α-globin genes (~ 95% α-thalassemias). Deletion of one or two α-genes is not associated with any symptoms. However, deletion of three α-genes or deletion of two α-genes in combination with the common α-globin mutation Hb constant spring results in Hb H disease (summarized in Table 8.2). Deletion of four α-genes is usually not compatible with life.

Although individuals harboring mutation of one (- α/α α) or two (--/α α) α-globin genes are asymptomatic, the child of asymptomatic parents with one and two α-globin gene deletions in cis, respectively, has 25% probability of inheriting Hb H disease (--/- α).

Hb H Disease This entity is characterized by accumulation of free β-globin chains. The free β-globin chains form homotetramers, known as Hb H (β4). Because of its instability, Hb H eventually precipitates in older red cells and makes inclusion bodies that lead to red cell breakdown and chronic hemolysis. Compensatory extramedullary erythropoiesis in the liver and spleen may lead to hepatosplenomegaly.

Laboratory Diagnosis High-performance liquid chromatography (HPLC) or capillary electrophoresis can detect the uncommon Hb H but is not helpful in common one or two α-globin gene deletions. Multiplex PCR/multiplex ligation-dependent probe amplification (MLPA) assay and, in some laboratories, globin chain synthesis assays are used for the detection of one or two α-globin genes deletions.

β-Thalassemias

β-Thalassemia most often results from minor single nucleotide changes resulting in alterations in promoter, initiation, splicing, or termination codons.

There are two classes of β-thalassemia: β^0, where there is complete absence of β-globin chains, and β+, where the production of β globin chain is diminished but not absent. Individuals who are heterozygous for β^0- or β+-mutation have one normal β-globin gene and are asymptomatic with mild anemia, also known as β-thalassemia minor. Subjects with homozygosity or compound heterozygosity for β^0-mutations produce no β-globin and have no Hb A and suffer from the most severe phenotype of β-thalassemia, also known as β-thalassemia major or Cooley's anemia. Compound heterozygosity or homozygosity for β+-mutations causes deficiency but not complete absence of β-globin, and these individuals have a relatively less severe phenotype (β-thalassemia intermedia) than that of β-thalassemia major.

Table 8.2 Alpha-thalassemia syndromes

	Syndrome	Mechanism	Genotype	Clinical manifestations
1	Silent carrier	Loss of one of the four α-globin genes	(– α/α α)	Clinically normal. Normal or slight decrease of MCV and Hb (~30% African-Americans)
2	Mild hematologic changes	Loss of two of the α-globin genes	(--/ α α; - α/- α)	Mild anemia, reduced cell Hb and MCV
3	Hemoglobin H disease	Loss of three of the four α-genes	(--/- α)	Varying degree of lifelong hemolysis, β-chains accumulate and make tetramers (β4 or Hb H)
4	Hydrops fetalis with Hb Barts	Loss of four α-genes	(--/--)	Incompatible with life mostly. Free gamma-chains make tetramers (Hb Bart). Severe tissue ischemia, heart failure, and generalized edema (hydrops fetalis)

Beta-Thalassemia Major (Cooley's Anemia) Anemia manifests after the first 6 months of life when gamma-globin production switches to β-globin production. Due to the absence of the β-globin chain, free α-globin chains accumulate. Precipitation of α-globin chains results in some breakdown of mature RBCs (hemolysis), but, more significantly, it causes breakdown of RBC precursors within the marrow (intramedullary hemolysis and ineffective erythropoiesis). β-Thalassemia major has a much more severe phenotype than Hb H disease. These patients are transfusion dependent, which further exacerbates their iron overload, the most common cause of death in these subjects. Cooley's anemia and the less severe β-thalassemia intermedia only rarely have significant hemolysis, and the major clinical effects of their pathophysiology are related to decreased Hb production and marrow expansion.

Laboratory diagnosis of β-thalassemia is made by HPLC or capillary electrophoresis, which invariably reveals increased Hb A2 (α2 δ2) and absent or diminished Hb A in β-thalassemia major and intermedia, respectively. β-Globin gene sequencing is done to identify the underlying β-globin mutation.

Sickle Cell Disease

Overview Sickle cell disease (SCD) is a chronic hemolytic anemia resulting from a point mutation in the β-globin gene (Hb S); it is an autosomal recessive disease. SCD is used to describe all of the conditions where RBC sickling occurs. Most of the pathophysiology in SCD is derived from vaso-occlusion rather than hemolysis (see Chap. 7).

Epidemiology SCD is most common in people of African descent; however, it is also seen in Mediterranean and Middle East people, South and Central Americans, and those of East Indian ancestry. Approximately 8% of African-Americans have the sickle cell trait (heterozygous form of Hb S), whereas approximately 1 in 600 are affected by sickle cell disease.

Pathophysiology The genetic defect in SCD is a point mutation in codon 6 of the β-globin gene (GTG for GAG) that results in the formation of Hb S. The mutation results in the substitution of a hydrophobic valine residue for a hydrophilic glutamic acid residue. Homozygosity for Hb S mutation is the most common form of SCD disease. SCD can also be caused by compound heterozygosity for Hb S and either a β-thalassemia mutation or one of several β-globin variants (e.g., Hb C, Hb Lepore, Hb E, $HbSD^{Punjab}$, and $HbSO^{Arab}$) that support Hb S polymerization. Hb S polymerizes on deoxygenation in microvascular beds, where hypoxia and acidosis induce Hb to release oxygen, increasing the intracellular concentration of the deoxygenated form of Hb S. The polymerization of Hb S induces deformation and rigidity of the RBC membrane and impairs transit of the SCD RBCs through the microvasculature. Although polymerization of Hb S is reversible after return of the RBCs to the arterial circulation, after multiple cycles of Hb S polymerization, RBCs become irreversibly damaged and trapped in microvascular beds, where they are lysed (intravascular hemolysis). Chronic hemolysis in SCD leads to release of free Hb. Free Hb produces reactive oxygen species and is also a potent scavenger of nitric oxide. Nitric oxide is responsible for vasodilatation and inhibition of adhesion molecule expression.

Clinical Presentation SCD is characterized by periodic episodes of acute vascular occlusion (painful crisis) that have their onset in the 1st or 2nd year of life. Painful crisis may be precipitated by events that impair tissue oxygenation, perfusion, or acid-base status. Infection, especially pneumonia, and systemic dehydration are common precipitating events; however, these non-hemolytic complications are not a subject of this chapter.

Patients with SC and HbS/β-thalassemia genotypes have a milder form of sickle cell disease than SS patients.

Laboratory Diagnosis Sickle cells are present on the blood film in all SCD genotypes. Hb S has an altered electrophoretic mobility and is detected on HPLC/capillary electrophoresis and confirmed by solubility assay.

Treatment of Sickle Cell Disease

Treatment for patients with sickle cell disease is supportive.

In young individuals with severe clinical disease and a matched sibling donor, allogeneic hematopoietic cell transplantation can be curative.

Because increased levels of Hb F inhibit Hb S polymerization, medications that increase gamma-globin synthesis may be useful in decreasing the frequency of acute painful crisis. Hydroxyurea, an inhibitor of ribonucleotide reductase, has been shown to increase Hb F levels and is approved for the treatment of patients with frequent painful crises. L-Glutamine was approved in 2017 to decrease the frequency of painful crises in adults and children. Penicillin prophylaxis and pneumococcal anti-infective vaccination should be given to children to prevent encapsulated bacterial infections.

Unstable Hemoglobins

Pathophysiology Unstable Hbs are an important group of clinically significant Hb variants. Several different mechanisms lead to the generation of unstable variants, which result in a congenital hemolytic anemia with Heinz bodies in RBCs (see G6PD deficiency above). Variant Hbs are found only in the heterozygous state.

Clinical Presentation Patients with unstable Hb variants have varying degrees of hemolytic anemia. This ranges from an asymptomatic hemolytic state to severe, life-threatening hemolysis. Typically, hemolysis is exacerbated by increased oxidant stress such as infections and the use of oxidant drugs. Patients may have splenomegaly.

Laboratory Diagnosis Since many unstable Hbs are not detected by Hb electrophoresis, the first test for this condition is one of the tests for Hb solubility, i.e., *isopropanol test* or *heat test*. Those unstable Hb mutants caused by β-globin gene mutations are not expressed at birth but become detectable at 3–6 months of age, while those caused by α-globin gene mutations are expressed since birth.

Treatment Supportive therapy only. There is no benefit from splenectomy.

Congenital Dyserythropoietic Anemias (CDAs)

CDAs are rare heterogeneous congenital dyserythropoietic anemias characterized by production of aberrant multinuclear erythroid progenitors. Erythroid hyperplasia is present. This is primarily a consequence of intramedullary hemolysis, but a minor component of aberrant hemolysis of mature RBCs may also be present.

Congenital Predisposition to Acute Microangiopathic Hemolytic Anemias

These are rare inherited disorders that may present at any age and are usually precipitated by an external event such as infection or pregnancy. Severe congenital deficiency (Upshaw-Shulman syndrome) or mutation of ADAMS13, a metalloprotease that cleaves large multimers of von Willebrand factor, predisposes to thrombotic thrombocytopenic purpura (TTP). In this disorder, red blood cells become sheared on long strands of von Willebrand factor. Atypical hemolytic uremic syndrome (aHUS) results from many different congenital mutations of components of the alternative complement pathway. In addition to other laboratory features of hemolysis, fragmented red blood cells (schistocytes) are discernable on blood smear. However, thromboses from aberrant platelet-vessel wall interaction are the principal feature of the morbidity of these disorders and are discussed elsewhere in this book (see Chap. 15).

Summary

Congenital hemolytic anemias result from heritable defects in components of the RBC membrane, metabolic pathways, or Hb. Important clues to the diagnosis may be obtained from the personal and family history and the peripheral blood smear. There is increasing evidence that even compensated hemolysis without anemia can lead to clinically significant iron overload.

Suggested Reading

1. Kaushansky K, Lichtman MA, Prchal JT, Levi M, Press OW, Burns LJ, Caligiuri MA, editors. Williams hematology. 9th ed. New York: McGraw Hill; 2015. ISBN 978-0-07-183300-4
2. Kaushansky K, Lichtman MA, Prchal JT, Levi M, Press OW, Burns LJ, Caligiuri MA, editors. Williams manual of hematology. 9th ed. New York: McGraw Hill; 2016. ISBN 978-1-259-64247-0

Acquired Hemolytic Anemias

9

Theodosia A. Kalfa

Definitions

Normal human erythrocytes have an average lifespan of 115 days. Hemolytic anemias arise when erythrocytes (red blood cells, RBCs) have decreased survival either due to an inherent abnormality of the cell (intrinsic or intracorpuscular defect) or due to extrinsic factors or both. Intrinsic defects include germ line (hereditary) genetic mutations causing hemoglobin disorders (defects in globin chains or heme synthesis), erythrocyte cytoskeleton disorders, and RBC enzymopathies. *Acquired hemolytic anemias* are most commonly due to extrinsic factors and include direct damage to RBC membrane induced by antibodies, toxins, or infectious agents; indirect mechanical injury to erythrocytes caused by abnormalities in the circulation, e.g., traumatic lysis due to vascular malformations or microangiopathic hemolytic anemia; or an overactive mononuclear phagocyte system, like in hypersplenism. As the exception to the rule, paroxysmal nocturnal hemoglobinuria (PNH) is a form of acquired hemolytic anemia due to a clonal genetic mutation.

1. *Hemolysis* in Greek means literally the rupture (lysis) of blood (hema) and refers to the premature destruction of RBCs, i.e., shortened RBC survival. The site of destruction may be *intravascular* (within the circulation) or *extravascular* (by the macrophages of the reticuloendothelial system of the liver and spleen). Hemolysis can also be episodic/acute or chronic.
2. *Anemia* is defined as a hemoglobin concentration below the normal range. It is more accurate to define anemia based on hemoglobin rather than on hematocrit or RBC count, since hemoglobin reflects more accurately the oxygen transport capacity of the blood and it is measured directly in CBC (complete blood count) instruments. Hematocrit, on the other hand, is a calculated value from the RBC number and MCV [RBCs (in 10^9/μl) × MCV (in fl) × 100/1000], except when a microhematocrit is obtained via centrifugation of a capillary tube containing whole blood. In the microhematocrit capillary tubes, hematocrit is actually measured as percent of the blood volume. The normal range of hemoglobin varies depending on age, gender, race, as well as the physiologic requirement for O_2 delivery to tissues, e.g., the normal level of hemoglobin would be elevated in patients with cyanotic congenital heart disease or chronic obstructive pulmonary disease or in individuals living at high altitude.

Hemolysis can occur and persist without anemia, in cases where increased RBC production triggered by increased erythropoietin compensates for the increased RBC destruction; however, most commonly this compensation is inadequate, resulting in hemolytic anemia, with symptoms, signs, and laboratory results reflecting both aspects of hemolysis and anemia.

Clinical and Laboratory Evaluation

Clinical Characteristics

Symptoms/signs of anemia (because of tissue hypoxia or compensatory mechanisms):

Fatigue, irritability, lethargy
Pallor
Dyspnea
Palpitations, tachycardia

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H. M. Lazarus, A. H. Schmaier (eds.), *Concise Guide to Hematology*, https://doi.org/10.1007/978-3-319-97873-4_9

Symptoms/signs of hemolysis

Scleral icterus, jaundice, dark urine
Gallstones (small, pigmented), cholecystitis
Splenomegaly
Leg ulcers

Acute intravascular hemolysis may be associated with fever, chills, backache, and signs of renal failure.

Medical history taking should investigate for complete drug history, recent travels, and exposure to infectious agents and toxins and include a comprehensive review of systems.

Laboratory Evaluation

Basic Tests to Evaluate Hemolytic Anemias

- *Complete Blood Count (CBC) with Reticulocyte Count*
  - Hemoglobin concentration establishes the degree of anemia, but increased reticulocyte count is the laboratory finding that indicates hemolysis: an increased reticulocyte count demonstrates that the anemia is due to increased destruction of RBCs and not due to decreased production. Reticulocytes are the young RBCs produced after enucleation of the orthochromatic erythroblasts in the bone marrow. A necessary first step for evaluation of anemias, the reticulocyte count, is characterized as the "poor man's bone marrow aspirate."
    Some exceptions may exist, having a hemolytic anemia without increased reticulocyte count, e.g.:
    - In cases of hyperacute hemolysis, the first 12–24 h. after the onset of disease
    - In rare cases where autoimmune hemolytic anemia is due to autoantibodies that may also be directed toward reticulocytes or RBC precursors
    - In extremely abnormal dyserythropoiesis when reticulocytes are being destroyed even before release from the bone marrow (usually hereditary cases of complete alpha-spectrin deficiency or congenital dyserythropoietic anemias and some acquired hemolytic anemias that present along with aplastic anemia (e.g., cases of PNH))
  - The absolute reticulocyte count (ARC) gives a better insight into the compensation mounted by the bone marrow as a response to hemolysis.
  - There is frequently slight macrocytosis indicated by increased mean corpuscular volume (MCV) of the RBCs, due to the increased cell volume of the reticulocytes. However, microspherocytes (small RBCs without central pallor) seen in chronic warm autoimmune hemolytic anemia (w-AIHA) or fragmented RBCs (schistocytes) seen in microangiopathic hemolytic anemia (MAHA), may offset the larger MCV caused by reticulocytosis, resulting to a final "normal" MCV.
- *Peripheral Blood Smear*
  - The normal mature RBC has a shape of biconcave disc; therefore, in the blood smear, it appears to have a central pallor which covers normally the 1/3 of the cell diameter.
  - The normal appearance of RBCs in the blood smear (Fig. 9.1) demonstrates minimal anisocytosis (variation in size) and minimal poikilocytosis (variation in shape).
  - Reticulocytes are recognized in the smear from their purple hue and usually larger size. Increased reticulocytosis is signified by polychromasia (meaning in Greek, multiple colors).

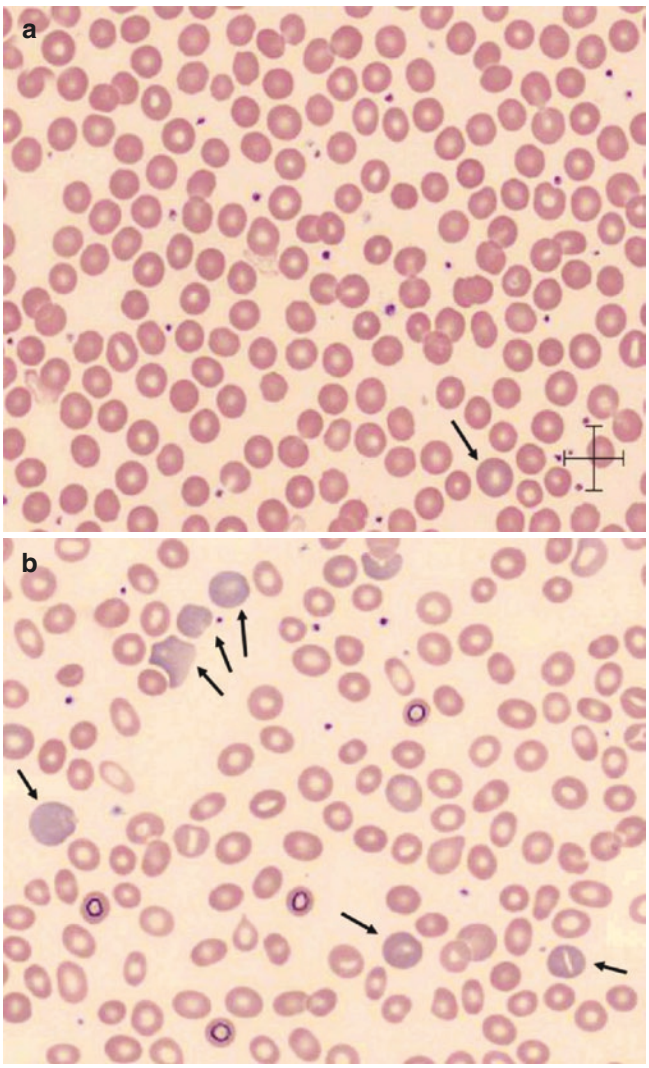

Fig. 9.1 (**a**) RBCs in a normal blood smear. Arrow points to a reticulocyte (larger cell with purple hue). (**b**) Blood smear of a patient with chronic autoimmune hemolytic anemia. Anisocytosis (variable cell size), poikilocytosis (variable cell shape), and polychromasia ("multiple colors" due to the presence of several reticulocytes with their purplish color) are prominent

- *Lactate Dehydrogenase (LDH)*
  - A sensitive, although not specific, indicator of hemolysis (this intracellular enzyme is released with any cell destruction). Typically increased with hemolysis, especially intravascular; it is a useful indicator to monitor along the course of the disease.
- *Bilirubin* is produced by the catabolism of the heme porphyrin molecule, and, after conjugation to a water-soluble form within the hepatocytes, it is excreted in the biliary system. In hemolysis, the unconjugated (indirect) bilirubin may be increased.
- *Serum aspartate aminotransferase (AST)* may be disproportionately higher than serum alanine aminotransferase (ALT) in hemolysis.
- *Haptoglobin* is produced in the liver and binds/scavenges free hemoglobin.
  - Decreases with hemolysis, frequently to undetectable levels, especially in intravascular hemolysis (Table 9.1).
  - It may remain normal, despite hemolysis, at times of acute inflammation since its production increases as an acute phase reactant.
- *Plasma-Free Hemoglobin*
  - Increases typically in brisk intravascular hemolysis and may result in pink plasma upon inspection of a microhematocrit capillary tube. It may be normal if its release does not exceed the capacity of haptoglobin to bind to it.
- *Urine Hemoglobin and Hemosiderin*
  - When plasma-free hemoglobin exceeds the capacity of haptoglobin binding, it passes through the renal glomeruli and is excreted with urine. Part of this excreted hemoglobin is absorbed by the proximal tubular cells where the iron is converted to hemosiderin. When these tubular cells are shed into the urine, typically 3–7 days after intravascular hemolysis, urine hemosiderin is detected in the urine sediment (Table 9.1).
- *Urine Urobilinogen*
  - A product of bilirubin reduction; it is increased with hemolysis or liver dysfunction.
- *Serum Iron, TIBC, Ferritin*
  - To evaluate for secondary iron deficiency that may follow chronic hemolysis with hemoglobinuria (e.g., PNH)

Table 9.1 Laboratory data discriminating between extravascular and intravascular hemolysis

		Extravascular	Intravascular
Plasma or serum	Haptoglobin	↓↓ or absent	Absent
	LDH	↑	↑↑
	Plasma Hgb	Normal or ↑	↑↑
Urine	Hemosiderin	0	↑
	Hemoglobin	0	↑

- *Serum and RBC Folate*
  - To evaluate for secondary folate deficiency that may follow chronic reticulocytosis.

Combination of extravascular and intravascular hemolysis may happen in several hemolytic anemias, especially the ones involving complement activation on the RBC surface. Complement cascade may reach either up to C3b, in which case the cells are phagocytosed by macrophages with C3b receptors in the liver, or up to membrane attack complex (MAC, i.e., C5b-9) leading to intravascular hemolysis.

In addition, ineffective erythropoiesis is practically hemolysis that takes place in the bone marrow, with positive hemolytic markers, reflecting premature destruction of erythroid precursors.

Special Tests to Explore the Cause of Hemolytic Anemia

Upon diagnosing hemolysis, immune-mediated mechanisms are coming first in the algorithms for differential diagnosis of the cause. It is important to diagnose autoimmune hemolytic anemia as soon as possible, since it may be fast evolving and life threatening with abrupt decrease of the hemoglobin, requiring immediate diagnosis and appropriate treatment. Hence, the first test to order for the work-up of a hemolytic anemia is DAT (direct antiglobulin test) and indirect antiglobulin test (Fig. 9.2).

- *Direct antiglobulin test (DAT; direct Coombs' test)*
  - Detects the presence of IgG or C3 complement bound to the RBC membrane.
  - Antibody or C3 complement titer can be determined, but this does not necessarily correlate with the severity of the hemolysis.
- *Indirect antiglobulin test (indirect Coombs' test)*
  - Detects the presence of antibodies against RBC membrane antigens in the serum of patients that may have already or are at risk of developing immune-mediated hemolysis. It is used to check for transfusion incompatibility (ordered as the "screen" in "type+screen") as well for low-affinity autoantibodies.
  - Additional testing is needed to differentiate between autoantibodies and alloantibodies.

A note has to be made that DAT can have a false-negative rate of 3–11%, in typical laboratories, especially for warm antibody autoimmune hemolytic anemia (w-AIHA). Such patients with DAT-negative AIHA may have IgA or monomeric IgM autoantibodies bound to RBCs, without fixation of complement, or a low number of antibodies bound on the RBC surface (<150) or low-affinity antibodies that are being removed during the wash phase of the assay. Hemolytic anemias with clinical characteristics of w-AIHA but with nega-

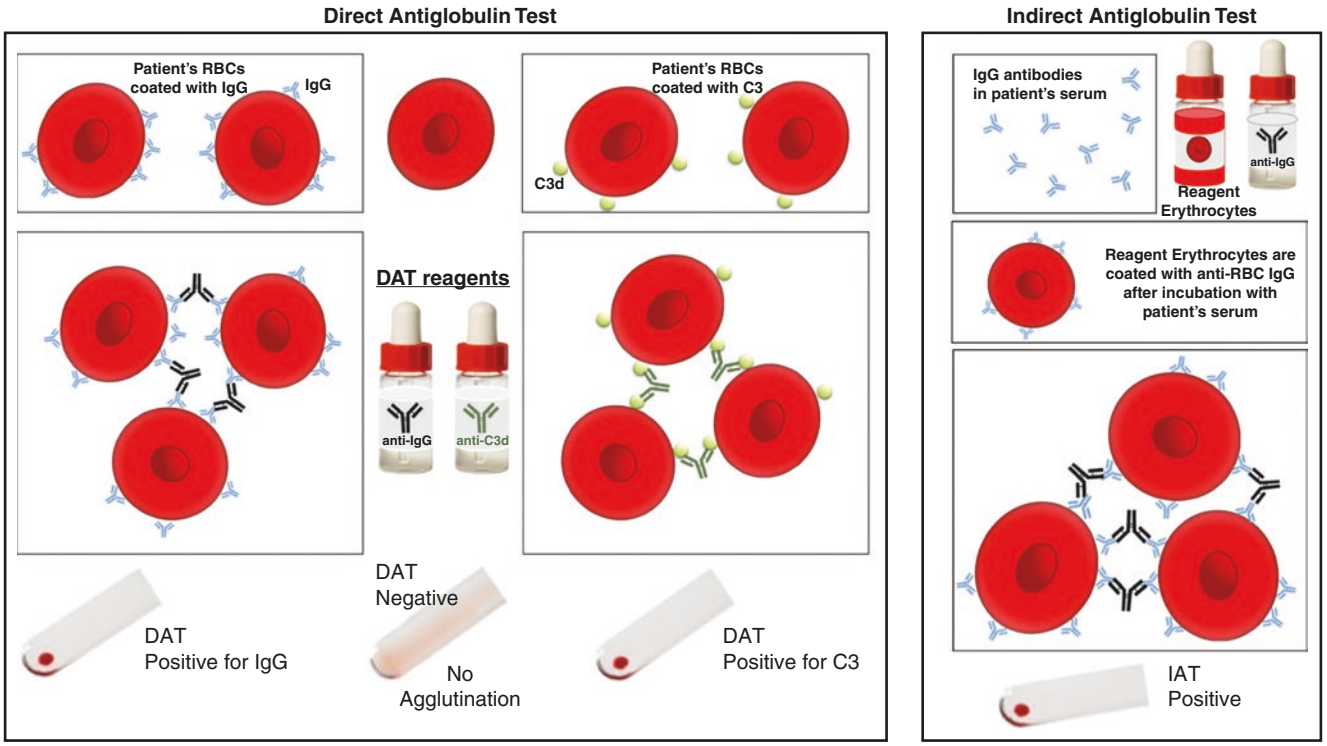

Fig. 9.2 The direct antiglobulin test (DAT) or direct Coombs' test evaluates for the presence of IgG or complement component C3 bound on the patient's RBCs, utilizing high-affinity anti-IgG or anti-C3 antibodies that cross-link the patient's RBCs when they are coated with IgG or C3 (reagents are available for C3b or C3d components of C3). A positive DAT is demonstrated by agglutination of the patient's RBCs in the test tube. The indirect Coombs' test (indirect antiglobulin test, orderable as "screen" when the order "type+screen" is placed) evaluates for the presence of unbound anti-RBC antibodies in the serum of the patient

tive DAT may have a positive result of "super Coombs" or "work-up for DAT-negative AIHA" performed in specialized referral laboratories, in about 50% of the cases.

- *Cold agglutinin titer*
  - Detects IgM autoantibodies that agglutinate RBCs at cold temperatures (4–18 °C).
  - Patient's serum is incubated in serial dilutions with normal RBCs at 4 °C, and the extent of RBC agglutination (clumping) present is "scored."
- *Peripheral blood flow cytometry for CD55 and CD59 (GPI-linked proteins)*
  - The diagnostic test for PNH, when the blood cell antigens CD55 and CD59 which normally protect cells from complement-mediated destruction are absent.
- *Donath-Landsteiner antibody test*
  - The diagnostic test for paroxysmal cold hemoglobinuria (PCH) caused by IgG autoantibodies that bind to RBCs and fix initial complement components at cold temperature and then activate complement at warm temperature.
  - The patient's blood is collected and kept at 37 °C until serum isolation. Then normal RBCs are incubated with the patient's serum at 0–4 °C (e.g., on ice) and then moved to 37 °C for a further incubation with fresh normal serum as an additional source of complement, resulting in hemolysis. Using RBCs from patients with PNH increases the sensitivity of the test since these cells are more prone to hemolysis by complement.
- *Bone marrow aspirate and biopsy*
  - Rarely needed in hemolytic anemia work-up; when performed they typically demonstrate erythroid hyperplasia.
  - May demonstrate dyserythropoiesis or megaloblastic changes with excessive or prolonged hemolysis.
  - Indicated to evaluate for bone marrow-infiltrating processes in work-up for splenomegaly or for bone marrow failure when clinical concerns in PNH.

Causes of Acquired Hemolytic Anemias

I. *Immune-mediated hemolysis*
(a) *Erythrocyte autoantibodies*
i. *Warm antibody autoimmune hemolytic anemia (w-AIHA)*
1. Idiopathic
2. Associated with primary immunodeficiency/immunodysregulation syndrome
3. Lymphoma or chronic lymphocytic leukemia (CLL)-associated
4. Secondary to other malignancies (rare)

5. Associated with systemic lupus erythematosus (SLE) or other collagen-vascular diseases
6. Secondary to viral infection
ii. *Cold agglutinin disease or syndrome due to IgM antibody*
iii. *Paroxysmal cold hemoglobinuria due to cold-reactive IgG antibody that fixes complement (=Donath-Landsteiner antibody)*
iv. *Drug-induced autoimmune hemolytic anemia*
1. α-Methyldopa type (inducing autoantibody to Rh antigens)
2. Penicillin type (stable hapten)
3. Quinidine type (unstable hapten)
(b) *Erythrocyte alloantibodies*
(a) Neonatal alloimmune hemolysis
(b) Posttransfusion hemolysis
i. Acute hemolytic reactions
ii. Delayed hemolytic reactions
II. *Traumatic hemolytic anemia*
(a) Impact
(b) Macrovascular defects-prostheses (such as mechanical heart valve)
(c) Microvascular causes
i. Disseminated intravascular hemolysis (DIC)
ii. Thrombotic thrombocytopenic purpura (TTP)
iii. Typical and atypical hemolytic uremic syndrome (HUS)
iv. Other microvascular abnormalities
III. *Hypersplenism*
IV. *Hemolytic anemia due to toxic effects on the membrane*
(a) Spur cell anemia
(b) External toxins
i. Animal or spider bites
ii. Metals
iii. Organic compounds
(c) Infectious agents
V. *Paroxysmal nocturnal hemoglobinuria (PNH)*

Some of the acquired hemolytic anemias are life threatening, e.g., severe autoimmune hemolytic anemia, thrombotic microangiopathy (TMA), or acute malaria. Prompt diagnosis and appropriate treatment is imperative.

It has to be recognized that sometimes hereditary hemolytic anemias may present late or remain unrecognized until adulthood; they should be considered in the differential of a hemolytic anemia at any age.

Outline per Disease Process

Immune-Mediated Hemolytic Anemia

An immune-mediated hemolytic anemia may be due to erythrocyte alloantibodies or due to development of autoantibodies against the RBCs.

1. *Erythrocyte Autoantibodies*
   i. *Warm Antibody Autoimmune Hemolytic Anemia (w-AIHA)*
      1. *Pathophysiology*
         - The autoantibodies react optimally with the RBCs at 37 °C.
         - They are usually IgG antibodies; rarely IgA or monomeric IgM.
         - The autoantibodies are polyclonal, and no autoantibody specificity can be identified: when eluted from the patient's RBCs for further testing, they are found to react with all common types of RBCs typically used in a blood bank test panel (pan-reactive).
         - Hemolysis is extravascular, in the spleen or liver.
           – The RBCs coated by warm-reacting autoantibodies are bound by spleen macrophages carrying Fc-γ receptors for the IgG heavy chain, and they are either phagocytosed or have part of their membrane removed (forming microspherocytes subject to further destruction during their next passage through the spleen).
           – High concentration of IgG or IgG with high affinity to complement on the RBCs may bind and activate complement up to C3b. C3b-opsonized RBCs are phagocytosed by liver macrophages that carry C3b receptors
      2. *Causes/Underlying Disorders*
         - Idiopathic
         - Associated with underlying immunodeficiency (e.g., CVID, ALPS, or AIDS)
         - Associated with lymphoma or chronic lymphocytic leukemia (CLL)
         - Secondary to other malignancies (rare)
         - Associated with systemic lupus erythematosus (SLE) or other collagen-vascular diseases
         - Secondary to viral infections
      3. *Clinical and Laboratory Findings*
         - Jaundice
         - Splenomegaly and occasionally hepatomegaly
         - Symptoms/signs associated with underlying disease
         - Anemia (decreased Hgb) with reticulocytosis, decreased or absent haptoglobin, elevated LDH, AST, and unconjugated bilirubin
         - DAT positive for IgG and frequently for C3
           – No autoantibody specificity can be identified; the autoantibodies react with all RBCs tested (pan-reactive).
           – Of note, there are cases of DAT-negative AIHA, e.g., due to IgA, monomeric IgM, low-affinity IgG, or low number of antibodies bound on the RBC surface. In ~50% of such cases, enhanced DAT work-up in referral laboratories may provide a positive result.
         - Peripheral blood smear: polychromasia, microspherocytes (Fig. 9.3)

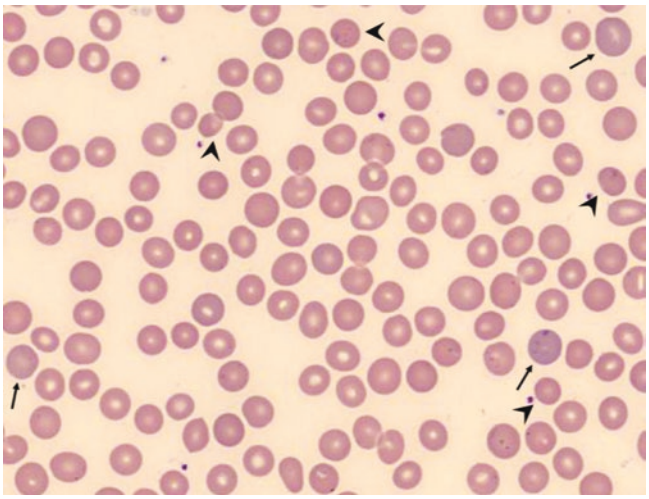

Fig. 9.3 Blood smear from a child with autoimmune hemolytic anemia and history of T1DM, demonstrating polychromasia (arrows point to reticulocytes) and microspherocytes, i.e., small RBCs with decreased central pallor (arrowheads). An exception to the rule, this patient had negative DAT and even negative enhanced DAT (super Coombs) but consistent evidence of hemolysis with increased LDH, unconjugated bilirubin, and non-detectable haptoglobin. Evaluation for PNH and other causes of hereditary or acquired hemolysis was negative and had no response to steroids, and transfusions failed to increase hemoglobin (indicative of immune-mediated destruction); ivIg increased both Hgb and reticulocytes (which are sometimes a target of autoantibodies). Response to ivIg proved that the patient had w-AIHA and MMF was initiated, stabilizing his Hgb

4. *Treatment Considerations*
   - First line
     - Glucocorticoids (if hemolysis improves with steroids, wean very slowly over at least 6 months; a fast wean has been associated with aggravated relapse of the disease).
     - Packed red blood cell (PRBC) transfusions
       Establish good communication with transfusion services to provide PRBC as needed to treat fast-progressing anemia. Since the patient has pan-reactive RBC antibodies, all donor units may be considered "incompatible"; even the patient's own RBCs are incompatible. Nevertheless, as hematologists say: "no patient should die of anemia."

       Transfusion services can typically evaluate for underlying alloantibodies that may have developed as a result of previous transfusions or pregnancies.

       An in vivo compatibility test may be useful when initiating a packed RBC transfusion in a patient with w-AIHA: rapid infusion of 20 mL blood followed by 20-min observation. If no reaction is noted, then the rest of the unit is given at the usual speed.
     - Immunoglobulin (IVIg), which blocks the Fc-γ receptors of spleen macrophages and potentially provides additional immune-modulatory effects
     - Plasmapheresis that physically removes free-circulating antibody
   - Second line
     - Splenectomy
     - Rituximab (anti-CD20 antibody)
     - Mycophenolate mofetil (MMF)
     - Sirolimus

   Use caution for cases of AIHA associated with immune dysregulation/immunodeficiency syndromes (e.g., ALPS)—in such cases splenectomy should be avoided, and rituximab may cause permanent B-cell depletion. MMF or sirolimus is preferable for ALPS and ALPS-like syndromes.
   - Third line
     - Azathioprine
     - 6-Mercaptopurine
     - Cyclosporine
     - Low-dose cyclophosphamide
   - Fourth line
     - High-dose cyclophosphamide
     - Alemtuzumab (Campath)
     - Bortezomib
     - Hematopoietic stem cell transplant (HSCT)

The two clinical entities of AIHA which are due to cold-reacting autoantibodies are defined by the immunoglobulin isotype against the RBCs: IgM in cold agglutinin disease or syndrome (CAS) and IgG in paroxysmal cold hemoglobinuria (PCH).

ii. *Cold Agglutinin Disease or Syndrome (CAD or CAS) due to IgM Antibody*

1. *Pathophysiology*
   - The autoantibodies are pentavalent IgM (i.e., cold agglutinins) directed against RBCs, usually against the i antigen, which is the straight-chain tetrasaccharide unit of the ABH molecules, or the I antigen, the branched version of these glycoproteins.
   - IgM autoantibodies are maximally reactive in the cold (4–18 °C) but may keep a reactivity up to at least 30 °C (wide "thermal amplitude"). They fix complement much more readily than IgG (attachment sites for C1q adjacently located within the same molecule).
   - Activation of the complement follows as IgM-bound RBCs circulate to the warm core temperature.
   - When C3b binds, RBCs are phagocytosed by the liver macrophages carrying C3b receptors.

- If the IgM titer is high, activation of complement may continue up to completion of the membrane attack complex, leading to intravascular hemolysis and a more acute and severe anemia.

2. *Causes/Underlying Disorders*
   - Acute CAD, more frequent in children and with a self-limited course, is typically post-infectious, with polyclonal IgM.
     – Anti-i is frequently associated with infectious mononucleosis (and less commonly with CMV or varicella).
     – Anti-I is typically seen with mycoplasma infections.
   - Chronic CAD or CAS, typically in middle-aged or elderly patients, due to monoclonal IgM (also most frequently directed against the i/I carbohydrate RBC antigens), is a difficult-to-treat paraneoplastic syndrome, secondary to lymphoma, chronic lymphocytic leukemia (CLL), Waldenström's macroglobulinemia, or rarely other malignancies.
3. *Clinical and Laboratory Findings*
   - Jaundice
   - Hepatosplenomegaly
   - Cold-induced acrocyanosis (i.e., blue discoloration of the fingertips, toes, nose, earlobes)
   - Symptoms/signs associated with an underlying disease
   - Anemia (decreased Hgb) with reticulocytosis, decreased or absent haptoglobin, elevated LDH, AST, and unconjugated bilirubin
   - DAT positive for C3 but not for IgG
   - Cold agglutinin titer positive
   - Agglutination of normal RBCs by the patient's serum at 4–18 °C (a "bedside" test on ice may also be performed)
   - Rouleaux formation indicating RBC agglutination is frequently noted on the blood smear (Fig. 9.4)
4. *Treatment*
   - Keep the patient warm.
   - Packed RBC transfusions as needed; transfuse blood utilizing a blood-warming device.

A notable feature of CAS is a high variability in hemolysis. Correspondingly, the need for transfusions varies greatly between patients.

- In children, CAD is typically a self-limited disease, requiring just supportive care. If steroids are started at presentation, before the diagnosis is clear, they can be weaned swiftly (in contrast to w-AIHA).
- In contrast, chronic CAS is difficult to manage. Splenectomy is not indicated since hemolysis mainly happens in the liver or intravascularly. Treatment considerations include:

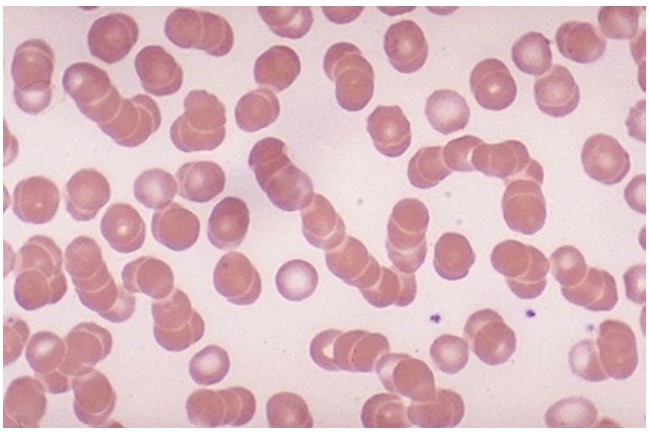

Fig. 9.4 Peripheral blood smear of a patient with cold agglutinin disease. Several RBC Rouleaux are seen

  – Treating the underlying condition
  – Corticosteroids (rarely effective as monotherapy)
  – Cyclophosphamide with prednisone or fludarabine regimens
    Rituximab (anti-CD20 antibody)
    Eculizumab (anti-C5 antibody)

In some unusual cases, considered as "mixed AIHA," the laboratory data satisfy the serologic criteria of both warm AIHA and CAS.

iii. *Paroxysmal Cold Hemoglobinuria (PCH) due to Cold-Reactive, Polyclonal IgG Antibodies that Fix Complement (=Donath-Landsteiner Antibodies)*

1. *Pathophysiology*
   - The autoantibodies (called Donath-Landsteiner antibodies) are polyclonal IgG directed against the P antigen of RBCs. These antibodies are biphasic hemolysins: after binding on RBCs, fix the initial complement components at cold temperatures, and then, upon warming to 37 °C, complete the complement cascade leading to intravascular hemolysis.
   - PCH typically has a good prognosis after remission; however, it can be life threatening on presentation due to severe and rapidly progressive anemia.
2. *Causes/Underlying Disorders*
   - PCH is usually post-infectious:
     – Tertiary or congenital syphilis used to be the most frequent cause of PCH, but this is now uncommon.
     – Now it is seen more frequently in children after or along an acute viral infection; the causative agent is often not identified.
     – Rarely it can be seen associated with autoimmune disorders in adults as a chronic condition.

3. *Clinical and Laboratory Findings*
   - Symptoms occur or are aggravated following cold exposure.
   - Fever and chills; back, leg, and abdominal pain.
   - Jaundice and pallor, fast progressing.
   - Red-brown urine due to hemoglobinuria.
   - Anemia (decreased Hgb) with reticulocytosis, decreased or absent haptoglobin, elevated LDH, AST, and unconjugated bilirubin.
   - DAT may be positive for C3 but not for IgG because the antibody either elutes from the cells during their preparation at room temperature or the antibody-coated cells are briskly lysed.
   - Donath-Landsteiner test may be performed by blood bank services.

The patient's blood has to be collected and kept at 37 °C until serum isolation. Then normal RBCs are incubated with the patient's serum at 0–4 °C (e.g., on ice) and then moved to 37 °C for a further incubation with fresh normal serum as an additional source of complement, resulting in hemolysis. Using RBCs from patients with PNH increases the sensitivity of the test since these cells are more prone to hemolysis by complement.

4. *Treatment*
   - Keep the patient warm.
   - Packed RBC transfusions as needed; transfuse blood utilizing a blood-warming device.
   - Supportive care.
   - Chronic autoimmune PCH may respond to prednisone or other immunosuppressants (azathioprine or cyclophosphamide). It does not respond to splenectomy, since the hemolysis is intravascular.

iv. *Drug-Induced AIHA*

1. α-Methyldopa type
   - The drug alters the endogenous Rh antigens, making them immunogenic; therefore, autoantibodies may be induced.
   - The autoantibody does not react with the drug.
   - Presentation resembles w-AIHA.
   - Hemolysis decreases over the course of several weeks after discontinuation of the drug.
2. Penicillin type
   - Stable hapten, forms a "tight" antigenic complex with a RBC membrane protein and induces IgG autoantibodies
   - Hemolysis occurs only when the drug is present
3. Quinidine type (examples include quinine, quinidine, sulfonamides, sulfonylureas, phenacetin, stibophen, and dipyrone)
   - Unstable hapten.
   - These drugs form a "loose" complex with RBC membrane glycoproteins, easily washed off during the washing steps of DAT.
   - Induce autoantibodies, IgG or IgM (the latter fixes and activates complement causing intravascular hemolysis).
   - DAT may be positive for IgG (when this is the autoantibody present) if drug is added to the incubation mixture. When the autoantibody is IgM, the DAT is negative for IgG but usually positive for C3.
   - Resolution follows fast after discontinuation of the drug.
2. *Erythrocyte Alloantibodies*

Alloimmune hemolytic anemia is more frequently seen in neonatal alloimmune hemolysis and rarely because of exposure to alloantibodies through transfused blood products.

i. *Neonatal Alloimmune Hemolysis (or Hemolytic Disease of the Newborn)*

1. *Pathophysiology*
   - IgG from the mother leaks into the fetal circulation during late pregnancy by transplacental passage or during delivery.
     – Due to incompatibility of Rh D antigen between mother and fetus:
       The D-negative mother is sensitized by D-positive red cells of the fetus escaping to her circulation.
       The titer of anti-D antibodies that the mother produces is enhanced with every pregnancy.
     – Due to incompatibility in the ABO blood group system (and more rarely in other RBC antigen systems):
       ABO incompatibility occurs typically when the mother is of blood group O and her infant of blood group A or B (more commonly group A).
       Relatively low numbers of group A or B antigens on neonatal red cells in comparison to the D antigens allow the antibody-coated cells to remain in the circulation for a longer period than in Rh D disease.
       There are a few case reports with group A mothers developing a high titer anti-group B IgG.
2. *Clinical and Laboratory Findings*
   - Hemolysis due to Rh alloimmunization is typically brisk and may happen in the fetal and neonatal period with:
     – Anemia reaching the severity of hydrops fetalis (anasarca edema and hepatosplenomegaly)
     – Hyperbilirubinemia that can cause, if not aggressively treated, kernicterus
     – DAT typically strongly positive for IgG
     – Pronounced stress erythropoiesis, indicated in the blood smear by nucleated RBCs and excessive reticulocytosis

- Hemolysis due to ABO incompatibility is usually much less severe with slowly developing mild anemia with reticulocytosis.
  - Microspherocytes are prominent in the blood smear with polychromasia but little or no increase in nucleated RBCs.
  - DAT may be weakly positive.
  - Indirect Coombs (IAT) is also positive due to not so strong affinity of anti-A or anti-B antibodies to the RBCs.
  - The severity of hyperbilirubinemia is exacerbated in neonates who also have G6PD deficiency (even the A^- form) and in those with Gilbert's syndrome.

3. *Treatment*
   - For Rh alloimmunization:
     - After the 1970s, prevention with anti-D polyclonal IgG is the standard of care in prenatal (third trimester) and post-delivery care for the Rh-negative mothers, making Rh D alloimmunization a rare incidence.
     - Affected neonates with Rh-associated hemolytic disease are likely to require exchange transfusion and be at risk for significant neonatal morbidity and mortality.
   - For neonatal alloimmune hemolysis due to ABO incompatibility:
     - Treatment depends on the severity of jaundice.
       Exchange transfusion is rarely required since intensive phototherapy is usually sufficient to control the hyperbilirubinemia.
       Simple PRBC transfusion for anemia is rarely required.

Of note, the severity of hyperbilirubinemia may be exacerbated in neonates with additional hereditary causes for neonatal hemolysis, e.g., *G6PD deficiency (even the A^- form, common in African-American boys) and in those with Gilbert's syndrome.*

- *Posttransfusion Hemolytic Reactions*
  1. Immune hemolytic transfusion reactions
     - Acute hemolytic reactions
       - Occur within 24 h of a RBC transfusion.
       - Due to mismatch/incompatibility of the patient with the donor RBCs, either because of human or machine error.
       - The recipient's antibodies against the donor RBCs are usually against the ABO system or less frequently against Duffy or Kell antigens.
       - Recipient antibodies bind the donor RBCs and
         May activate complement causing intravascular hemolysis
         May opsonize the donor RBCs leading to their sequestration and phagocytosis by macrophages of the liver and spleen

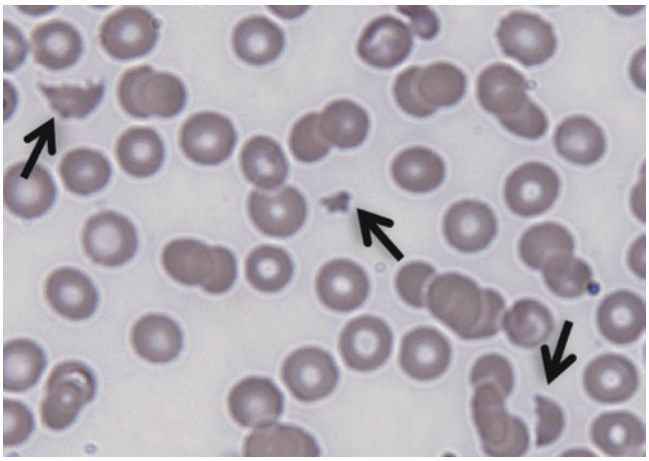

Fig. 9.5 Schistocytes (meaning "torn-apart cells" in Greek) are pointed by arrows in the blood smear of a patient with atypical hemolytic uremic syndrome

- *Treatment*
  i. Stop the transfusion if still ongoing.
  ii. Supportive care.
  iii. Aggressive hydration to maintain good urine output and prevent renal failure in case of brisk intravascular hemolysis and massive hemoglobinuria.
     - Delayed hemolytic reactions
       - Occur usually ~2 weeks up to 30 days post-PRBC transfusion.
       - Due to an amnestic response of the immune system (IgG antibodies) to a foreign red blood cell antigen from previous exposure, for example, pregnancy or previous transfusions.
       - Typically, they lead to extravascular hemolysis, and they are mild, even clinically silent, and typically self-limited. Alternatively, these patients respond to a course of prednisone.

  2. Nonimmune hemolytic transfusion reactions
     - Due to thermal, osmotic, or mechanical injury of the transfused red blood cells, usually causing intravascular hemolysis

Traumatic Hemolytic Anemia

RBCs are fragmented by mechanical trauma in the circulation with subsequent intravascular hemolysis. The hallmark of traumatic hemolytic anemia is the presence of schistocytes (fragmented cells) on the peripheral blood smear (Fig. 9.5).

1. *External Impact*
   - Hemoglobinemia and hemoglobinuria described in long-distance runners without appropriate shoes, following karate, or even after playing of bongo drums

2. *Macrovascular Defects-Prostheses*
   - Due to shear stress in patients with artificial valves or after aortofemoral bypass and in some patients with severe calcific aortic stenosis
3. *Microangiopathic Hemolytic Anemia (MAHA) or Thrombotic Microangiopathy (TMA)* (see Chaps. 13 and 15)
   - When RBCs are exposed to shearing forces circulating through fibrin and platelet microthrombi deposited in arteriolar sites
     i. *Disseminated Intravascular Hemolysis due to Disseminated Intravascular Coagulation (DIC)*
        1. Associated with an underline illness.
        2. Variable degree of anemia with reticulocytosis (typically mild or absent), thrombocytopenia, increased LDH, while PT/PTT and D-dimer are increased and fibrinogen is decreased, indicating a parallel consumption of clotting factors.
        3. Will improve with successful treatment of underlying disease.
     ii. *Thrombotic Thrombocytopenic Purpura (TTP)*

Historically, TTP had a mortality rate up to 90% when left untreated. Prompt recognition and initiation of early therapy have drastically reduced mortality rate to 10–20%.

Therefore, "if you think that the patient may have TTP, go ahead and treat for it."

1. *Pathophysiology*
   i. Due to deficiency or defect of ADAMTS-13, which is a cleaving protease that normally breaks down large multimers of von Willebrand factor (vWF).
   ii. Without ADAMTS-13, accumulation of vWF multimers (long strands of unprocessed vWF) attracts and binds platelets, resulting in subsequent thrombi in the microvasculature of the body, contributing to shearing of RBCs, and ultimately to end-organ damage.
   iii. Deficiency of ADAMTS-13 may be either inherited (rare; usually presents early in life) or acquired through an autoimmune mechanism.
2. *Causes/Underlying Disorders*
   i. Frequently, no inciting trigger can be identified.
   ii. TTP of autoimmune etiology may be associated with pregnancy, AIDS, SLE, scleroderma, or Sjögren's syndrome. In these cases, most commonly an autoantibody to ADAMTS13 induces the illness.
3. *Clinical and Laboratory Findings*
   i. Hemolytic anemia with RBC fragmentation and signs of intravascular hemolysis
   ii. Thrombocytopenia
   iii. Neurological findings: non-focal (confusion, delirium, altered state of consciousness up to coma) and/or focal (seizures, hemiparesis, aphasia, visual field defects)
   iv. Decreased renal function
   v. Fever

Although the above are referred as the "pentad" of symptoms in TTP, only 5–40% of patients have this classic presentation. A high index of suspicion is needed to make an early diagnosis before the "pentad" becomes manifest.

   vi. Nausea, vomiting, diarrhea, abdominal pain, mild to severe weakness, bleeding.
   vii. Variable degree of anemia with reticulocytosis, thrombocytopenia, increased LDH, while coagulation tests (PT/PTT, fibrinogen, D-dimer, fibrin split products) are usually normal or only mildly abnormal.
   viii. Death in the acute illness is commonly due to myocardial infarction; hence surveillance for cardiac injury is important.

4. *Treatment*
   i. Plasmapheresis daily for TTP due to ADAMTS-13 inhibitor (wean slowly over weeks or months) or plasma transfusions for TTP due to ADAMTS-13 deficiency.
   ii. Do not transfuse platelets (unless addressing life-threatening bleeding) since they precipitate thrombotic events ("do not throw oil to the fire").
   iii. Supportive care and PRBC transfusions may be required.
   iv. Rituximab or other immunosuppressive therapy (e.g., cyclophosphamide and prednisone, MMF, cyclosporine) is used for relapsing or refractory cases with promising results when antibodies to ADAMTS13 are detected (80–90% of cases).
      iii. *Hemolytic Uremic Syndromes: HUS and Atypical HUS (aHUS)* (see Chap. 15)

HUS and aHUS are also MAHA syndromes presenting with thrombocytopenia and microvascular thrombosis, typically with prominent renal impairment or failure.

1. *Pathophysiology*
   i. The typical HUS is due to Shiga toxin effects
   ii. Atypical HUS (aHUS) is:
      1. Due to excessive activation of the alternative pathway of the complement system
      2. Usually associated with mutations in the genes encoding complement regulatory proteins (including factor H, membrane cofactor protein (MCP), factor I, or thrombomodulin) or the genes C3 convertase proteins (C3 and factor B)
      3. May arise due to anti-factor H antibodies associated with a common polymorphism in complement factor H
2. *Causes/Underlying Disorders*
   i. Typical HUS is secondary to gastroenteritis due to Shiga toxin-producing bacteria, most frequently *Escherichia coli* O157:H7.

ii. aHUS may be triggered by:
   1. An infectious event, mainly upper respiratory tract infection or diarrhea/gastroenteritis (studies show that post-diarrheal onset does not eliminate the diagnosis of aHUS)
   2. Pregnancy or postpartum period
   3. Posttransplant

3. *Clinical and Laboratory Findings*
   i. Typical HUS has a prodrome of gastroenteritis with bloody diarrhea.
   ii. aHUS presents as HUS without bloody diarrhea, although a few cases of aHUS have been described to be triggered by gastroenteritis.
   iii. Hemolytic anemia with RBC fragmentation and signs of intravascular hemolysis.
   iv. Thrombocytopenia.
   v. Renal impairment (increasing creatinine).
   vi. Variable degree of anemia with reticulocytosis, thrombocytopenia, increased LDH, while coagulation tests (PT/PTT, fibrinogen, D-dimer, fibrin split products) are usually normal or only mildly abnormal. aHUS may be associated with abnormal D-dimer on presentation that ameliorates quickly with anti-C5 therapy.
4. *Treatment*
   i. Supportive care, including packed RBC transfusions as needed, hypertension control, hemodialysis when clinically indicated.
   ii. Plasmapheresis used to be recommended for aHUS; however it is associated with up to 8% mortality during the first episode and progression to end-stage renal failure; eculizumab, a humanized monoclonal antibody that blocks complement C5, is the preferred treatment.
   iv. *Other Microvascular Abnormalities Causing MAHA*
      1. Malignant hypertension
      2. Vasculitis
      3. Giant cavernous hemangiomas (Kasabach-Merritt syndrome)
      4. Renal allograft rejection
      5. Disseminated cancer
      6. Eclampsia/HELLP (hemolysis, elevated liver enzymes, and low platelets) syndrome
      7. Severe burns causing microvascular injury

Hypersplenism

Functional state of splenic hyperactivity, often but not always associated with splenomegaly, causing sequestration of blood cells and, subsequently, a shortened RBC, WBC, and platelet lifespan

Due to:

1. Vascular congestion
   - Hepatic fibrosis and/or cirrhosis
   - Portal vein thrombosis
   - Hepatic vein thrombosis (Budd-Chiari syndrome)
   - Splenic vein thrombosis
   - Right heart failure (long standing)
2. Extramedullary hematopoiesis
   - Thalassemias
   - Other hereditary hemolytic anemias (e.g., severe HS or PK deficiency)
   - Osteopetrosis
3. Infections
   - Viruses (CMV, EBV)
   - Bacteria
   - Tuberculosis
   - Fungi
   - Parasites (including malaria and toxoplasmosis)
4. Neoplastic infiltration
   - Lymphomas
   - Leukemias
   - Myeloproliferative disorders
   - Histiocytosis
   - Metastatic neoplasm
5. Inflammatory diseases
   - Rheumatoid arthritis
   - Systemic lupus erythematosus
6. Storage disorders
   - Lipid storage disorders (e.g., Gaucher disease)
   - Glycogen storage diseases
   - Mucopolysaccharidoses
   - Sarcoidosis
   - Amyloidosis
7. Nonmalignant structural abnormalities
   - Cysts
   - Hamartomas

Treatment

i. Treat underlying cause of hypersplenism/splenomegaly.
ii. Splenectomy only for cases of severe anemia and if benefits outweigh the risks of increased sepsis susceptibility and increased thrombophilia.

Hemolytic Anemia due to Toxic Effects on the Membrane

1. External toxins
   i. Animal or spider bites
   ii. Metals, e.g., copper excess with Wilson's disease
   iii. Drug-induced oxidative damage
   iv. Infectious agents, e.g., malaria, babesia

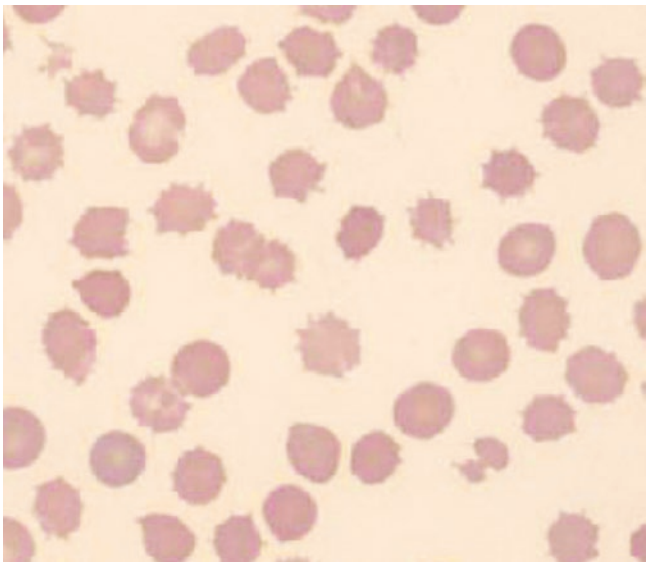

Fig. 9.6 Spur cells (RBCs with multiple spicules, irregular both in length and their spacing), and bizarre-shaped fragments in the blood smear of a patient with liver failure and spur cell anemia. The condition resolved after liver transplantation. Spur cells are also seen in patients with abetalipoproteinemia; however, that condition is associated with only mild hemolysis

2. Spur cell anemia
   - Hemolytic anemia with bizarre-shaped spiculated RBCs (spur cells—Fig. 9.6), associated with advanced cirrhosis.
   - The membrane of the spur cells has increased cholesterol but a normal phospholipid content and severely decreased survival; the cause is abnormal LDL carrying increase molar ratio of unesterified cholesterol to phospholipids.
   - The hemolysis happens in the spleen, and splenomegaly is always present.
   - Transfused RBCs are promptly altered to spur cells; therefore, PRBC transfusions do not help with spur cell anemia.
   - The condition is ameliorated by splenectomy (potential risks of increased thrombophilia post splenectomy need to be considered) and cured by liver transplantation

Paroxysmal Nocturnal Hemoglobinuria (PNH)

PNH is an acquired hemolytic anemia due to an intracorpuscular defect as a result of a clonal mutation of the PIGA gene at the level of hematopoietic stem cells.

1. *Pathophysiology*
   i. Acquired clonal disease.
   ii. It is a "complementopathy," like aHUS and cold agglutinin disease (CAD), caused by excessive complement activation.
   iii. Inciting event is an inactivating mutation of *PIGA*, a gene in the X-chromosome, encoding an enzyme important for the biosynthesis of the glycosylphosphatidylinositol (GPI) anchor.
   iv. The *PIGA* mutant hematopoietic stem cell (HSC) and all the blood cells derived from it miss GPI-anchored glycoproteins, like CD55 and CD59.
   v. CD55 and CD59 are complement regulatory proteins: CD55 inhibits the C3 convertase, and CD59 inhibits formation of the membrane attack complex (MAC); therefore, without CD55 and CD59, complement is overactivated leading to RBC lysis.
2. *Causes/Underlying Disorders*
   i. Typical HUS is secondary to gastroenteritis due to Shiga toxin-producing bacteria, most frequently *Escherichia coli* O157:H7.
   ii. aHUS may be triggered by:
      - An infectious event, mainly upper respiratory tract infection or diarrhea/gastroenteritis (studies show that post-diarrheal onset does not eliminate the diagnosis of aHUS)
      - Pregnancy or postpartum period
      - Posttransplant
3. *Clinical and Laboratory Findings*
   i. Intravascular hemolysis (fatigue, dyspnea, hemoglobinuria)
      - Hemolysis is chronic in PNH.
      - It is exacerbated in inflammatory states, pregnancy, and infections, when there is typically increased complement activation.
      - Iron deficiency (due to hemoglobinuria and hemosiderinuria) may exacerbate anemia.
   ii. Thrombosis (the leading cause of death in PNH)
   iii. Aplastic anemia
      - PNH may occasionally be diagnosed after transformation to myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML).
   iv. Peripheral blood smear demonstrates non-specific hemolytic anemia with polychromasia but normal RBC morphology.
   v. Tests used in the past for PNH diagnosis (not used clinically any more) are:
      - Ham's test: the patient's RBCs are incubated in acidified serum (lower pH activates complement). PNH RBC's are sensitive to complement lysis.
      - Sucrose lysis test: normal donor (fresh) serum is incubated with an isotonic sucrose solution, which activates complement. Lysis of the RBCs (indicating that they are sensitive to complement lysis) reveals PNH.
   vi. The preferred diagnostic study is flow cytometry for CD55 and CD59 in peripheral blood cells.
   vii. Bone marrow aspiration and biopsy may demonstrate a hypercellular bone marrow in response to hemolysis or may demonstrate bone marrow failure (aplastic

anemia). Aplastic anemia work-up should always include flow cytometry testing for PNH.

4. *Treatment*
   i. Eculizumab, a humanized monoclonal antibody, blocks terminal complement activation by binding to C5.
      - It sterically prevents C5 from binding to convertases to be cleaved into C5a and C5b and, therefore, compensates for the CD59 deficiency and inhibits the membrane attack complex (C5b-9 or MAC) formation and associated brisk intravascular hemolysis.
      - It does not compensate for the loss of cell surface CD55 allowing C3 fragment deposition on the PNH red cells and mild to moderate extravascular hemolysis.

   ii. Supportive care with PRBC transfusions, folic acid, iron supplementation as needed.

   iii. Anticoagulants (LMWH, warfarin, aspirin) for thrombosis.
      - Many PNH patients may be able to discontinue anticoagulation if their PNH is well controlled with eculizumab.

   iv. Allogeneic hematopoietic stem cell transplant for bone marrow failure.

Suggested Reading

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Physiology of Hemostasis

10

Alvin H. Schmaier

The Hemostatic System

(A) *Overview*

Development of much of our understanding of the hemostasis and thrombosis protection systems in vivo derived from translation of clinical observations seen in patients with unique biochemical and genetic processes. More recently mutant mice deleted of specific proteins in this system have additionally contributed to our understanding.

1. *Hemostasis*, the cessation of bleeding, occurs within the intravascular compartment lined with endothelium. Normal hemostasis and thrombosis (the occlusion of a blood vessel) is the sum of activity of two components: (1) a cellular part that consists of circulating cells and the vessel wall and (2) a protein portion from the blood plasma or cells in the intravascular compartment. Some patients have normal blood coagulation proteins but abnormal hemostasis due to a platelet defect (e.g., Bernard-Soulier syndrome; see Chap. 14). Alternatively, other patients have normal platelet and vessel wall function but abnormal hemostasis due to a blood coagulation protein defect (e.g., hemophilia A; see Chap. 12).
2. *Components*. The hemostatic/thrombotic system consists of both cellular and protein components that closely interact. The cellular components consist of leukocytes (neutrophils and monocytes), platelets, and endothelium. It is still not clear what cellular component provides the initial event in hemostasis and if it is the same under different circumstances. Platelets, anucleate cell fragments, historically have been considered the initial nidus upon which hemostatic reactions occur. This notion has led to the idea that platelets initiate primary hemostasis and on or about the locus of the platelet plug, blood coagulation protein reactions occur (secondary hemostasis). However, in certain models of hemostasis, vessel wall clot occurs first and on or about this formed fibrin, platelets are recruited into a dense and later loose platelet plug [1]. Adding complexity to the systems, vessel walls themselves constitutively are anticoagulant surfaces which when injured become procoagulant [2]. Monocytes and neutrophils contribute tissue factor initiating hemostasis both in arteries and veins and have potent clot-lysing system. The protein components that contribute to hemostasis and thrombosis include three protein systems: the blood *coagulation* (clot-forming), the *fibrinolytic* (clot-lysing), and *anticoagulant* (regulating) protein systems. Each of the proteins in these three systems balances the activities of the others.
3. *Regulation*. Physiologic hemostasis is a tightly regulated balance between the formation and dissolution of hemostatic plugs by the *coagulation and fibrinolytic systems*, respectively. Blood coagulation proteins circulate as zymogens or proenzymes and are not activated. When a stimulus/injury occurs, the proenzymes of the system are activated to enzymes initiating a series of proteolytic reactions that lead to thrombin formation, the main clotting enzyme. [Note: the convention in the coagulation protein field is to indicate a proenzyme (zymogen) as a Roman numeral and its active enzyme with the small letter "a" after the Roman numeral]. The blood coagulation proteins become activated in an apparent cascade-like fashion. The *anticoagulation* proteins regulate the coagulation and fibrinolytic systems. The proteins of the anticoagulation system join those of the fibrinolytic system to prevent or counterbalance coagulation reactions. Thus, the hemostatic system is tightly modulated by a

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series of serine proteases (enzymes), their cofactors for activity, and serine protease inhibitors that regulate their function. All these events occur on or about cells in the intravascular compartment that have their own levels of regulation.

(B) *Process*. A number of events are involved in hemostasis.

1. *Overview of the role of blood coagulation proteins in hemostasis*: When a vessel wall is injured, several events occur simultaneously. Von Willebrand factor helps flowing platelets to adhere to the vessel wall. Collagen in the vessel wall, now exposed, allows platelets to adhere also by their collagen receptors leading to activation. Platelet activation leads to thrombin formation on or about the platelet surface. Alternatively, before, simultaneously with or independent of platelet adherence depending upon the circumstance, vessel injury leads to exposure of subendothelial tissue factor (TF) along with factor VIIa which activates factor IX to factor IXa. Factor IXa activates factor X to factor Xa that leads to thrombin formation. Thrombin proteolysis of fibrinogen forms fibrin, which is the protein basis of a clot. Thrombin also recruits more platelets to the site of injury to initiate or enhance a platelet thrombus. It is recognized that in vivo, several pathways lead to thrombin formation. Similarly, when injured, endothelium too becomes a procoagulant surface. The procoagulant nature of endothelial cells is due to increased expression of TF and factor VIIa to initiate thrombin formation, increased synthesis of factor V to serve as a cofactor for more thrombin formation, inactivation of thrombomodulin for protein C activation, and increased plasminogen activator inhibitor expression with reduced tissue plasminogen activator.
2. *Overview of anticoagulation and fibrinolysis in hemostasis*. Circulation anticoagulants include antithrombin, tissue factor pathway inhibitor, and protein C. Under resting conditions, endothelial cells provide an anticoagulant surface. The anticoagulant nature of the endothelial cell membrane consists of a number of entities: antithrombin that inhibits all coagulation enzymes (see below); thrombomodulin for protein C activation (see below); tissue plasminogen activator release that stimulates fibrin clot lysis; nitric oxide and prostacyclin that stimulate induced vasodilation and inhibit platelets, respectively; and membrane-associated ectoADPases, CD39, that degrade ADP to limit platelet activation [3]. Prostacyclin also has an ability to downregulate vessel tissue factor through a Sirt1- and KLF4-mediated mechanism [4].

Coagulation Protein System

(A) *Coagulation Proteins* (Table 10.1)

Blood coagulation proteins can be grouped into three categories:

1. *Phospholipid-bound proenzymes (zymogens) of the vitamin K proteins* make up the physiologically essential hemostatic system.
   (a) These proteins are vitamin K-dependent and are synthesized in the liver. Vitamin K is required for an essential γ-carboxylation reaction that takes place on each of these proteins' Gla residues located on their amino terminal ends, making them an α-carboxyglutamic acid. This carboxylation reaction allows these proteins to bind to lipid and cell membranes, where they are activated. Without this carboxylation reaction, these proteins do not function normally by not assembling on membrane phospholipids. Inhibition of the carboxylation reaction is the basic mechanism on how the common oral anticoagulant warfarin works.
   (b) These proenzymes (zymogens) include factor VII, factor IX, factor X, and factor II (prothrombin). These proteins are essential for normal blood coagulation hemostasis and life. A complete deletion of factors VII, X, or II leads to lethal hemorrhage in utero or at the time of delivery. Factor IX deficiency is hemophilia B, one of the most severe bleeding states that survives gestation and delivery.
2. *Surface-bound proenzymes (zymogens) of the contact activation system*. This additional system to initiate hemostasis is not considered physiologic but participates in disease states and, independent of hemostasis, has a role in thrombosis.
   (a) Surface-bound proenzymes include factor XII (Hageman factor) (FXII), prekallikrein (Fletcher factor) (PK), and factor XI (FXI). The terms Hageman and Fletcher factor were used to name

Table 10.1 Proteins of the plasma blood coagulation system

Phospholipid-bound zymogens of the vitamin K-dependent protein system	Surface-bound zymogens of the contact activation system	Cofactors and substrates of the enzymes of the blood coagulation system
Factor VII	Factor XII	Tissue factor
Factor IX	Prekallikrein	Factor VIII
Factor X	Factor XI	Factor V
Factor II		Fibrinogen
		High M_r kininogen[a]

[a]M_r, Molecular weight

these proteins based upon the first patients recognized with the protein deficiency. Factor XI deficiency is associated with clinical bleeding; factor XII or prekallikrein deficiency is not. Factor XII levels, however, influence thrombosis (see sections "The Anticoagulation System" and "Cohesive Hypotheses for the Initiation of the Hemostatic System" below). However *all* of these proteins influence the common blood coagulation screening test called the activated partial thromboplastin time (aPTT). This test and its interpretation will be discussed in detail in Chap. 11.

(b) These protein zymogens are also known as the "contact proteins" because factor XII autoactivates when associated with a negatively charged surface (e.g., a glass tube in vitro or collagen, aggregated protein, RNA, DNA, or inorganic polyphosphate (polyP) released upon platelet activation or bacterial or human cell destruction in vivo). Autoactivation is the process whereby a coagulation protein zymogen when bound to a surface has a structural change such that a proenzyme changes an active enzyme. The molecular basis for this event has not yet been fully explained for factor XII.

3. *Hemostatic cofactors and substrates* of the enzymes of the coagulation system facilitate coagulation enzyme activity (see below in the next section).
   (a) *Tissue factor* (TF) is an essential cofactor for activated factor VIIa. It is found in most tissues and cells. Its synthesis is upregulated in inflammatory and injury states. Upregulation of TF results in the formation of complexes with factor VII that produces the initiation of hemostatic reactions. The absence of tissue factor is incompatible with successful mammalian gestation leading to intrauterine death.
   (b) *Factor VIII* (antihemophilic factor) is a cofactor that greatly facilitates the ability of the enzyme factor IXa to activate factor X in "tenase" (see below). Its absence is associated with the most severe clinically recognized bleeding disorder that survives gestation and delivery, hemophilia A.
   (c) *Factor V* (proaccelerin) is a cofactor that facilitates the ability of the enzyme factor Xa to activate factor II (prothrombin) to factor IIa (thrombin) in "prothrombinase" (see below). Its deficiency is associated with death from intrauterine hemorrhage or at delivery.
   (d) *Fibrinogen* is the main substrate of thrombin (factor IIa). When fibrinogen is proteolyzed by thrombin, fibrin monomer is formed. Fibrin monomer associates end to end and side to side to become insoluble and cross-linked to form a fibrin mesh that is an actual clot (thrombus). Severe deficiencies of fibrinogen survive gestation and delivery.

   Once formed, stability of the fibrin clot is produced by an enzyme called *factor XIII*, a tissue transglutaminase that cross-links the strands of associating fibrin to make a stronger insoluble structure. Factor XIII is like mortar and stabilization rods in a brick wall.
   (e) *High-molecular-weight kininogen* (Fitzgerald or Williams factor) (HK) is a cofactor for the activation of all the contact system proenzymes (zymogens), factor XII, prekallikrein, and factor XI. High-molecular-weight kininogen also is a substrate of the activated forms of the contact system enzymes to liberate a biologically active peptide called bradykinin. Bradykinin stimulates nitric oxide and prostacyclin formation in endothelial cells to produce vasodilation and platelet inhibition and reduce vessel wall tissue factor. Deficiency of high-molecular-weight kininogen is not associated with bleeding, but it delays induced arterial thrombosis.

(B) *Critical Protein Assemblies in Hemostatic Reactions*. The essential proteins of the blood coagulation system were identified by observation of patients, and the first recognized defect was named for the patient (e.g., Stuart factor, factor X deficiency). Deficiencies in coagulation factors VIII and IX are the most severe bleeding disorders that occur in patients who survive gestation and birth. The rare patients who have congenital deficiencies of coagulation factors VII, X, V, and II usually do not have severe bleeding states because these individuals must have some small amounts of functional coagulation factor to have survived gestation and birth. Directly or indirectly, all of these proteins participate in two critically important assemblies, "tenase" and "prothrombinase" that are essential for kinetically fast blood coagulation protein activation for thrombin generation. The tenase and prothrombinase assemblies are important because they are anticoagulant targets.

1. *Tenase assembly* (Fig. 10.1) is the ability of activated factors VIII and IX to assemble on phospholipid surfaces or cell membranes to accelerate the activation of factor X to factor Xa. When all of these components are present, the rate of factor X activation by factor IXa (i.e., catalytic efficiency) is increased a billion-fold, 1×10^9 faster over the rate of factor IXa activation of factor X alone. This fast rate is what makes it physiologic and an important juncture point in the system.

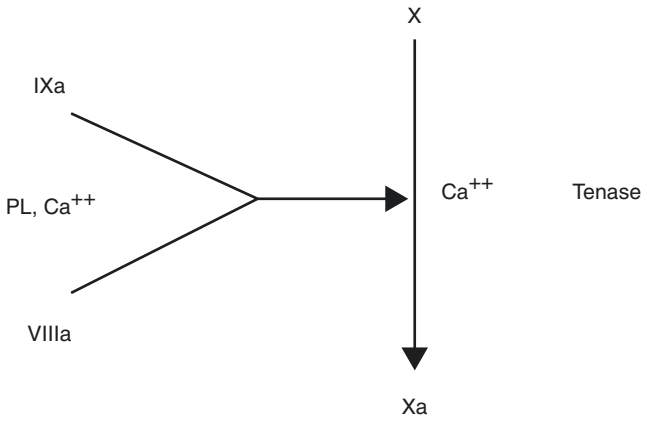

Fig. 10.1 The tenase assembly for factor Xa formation

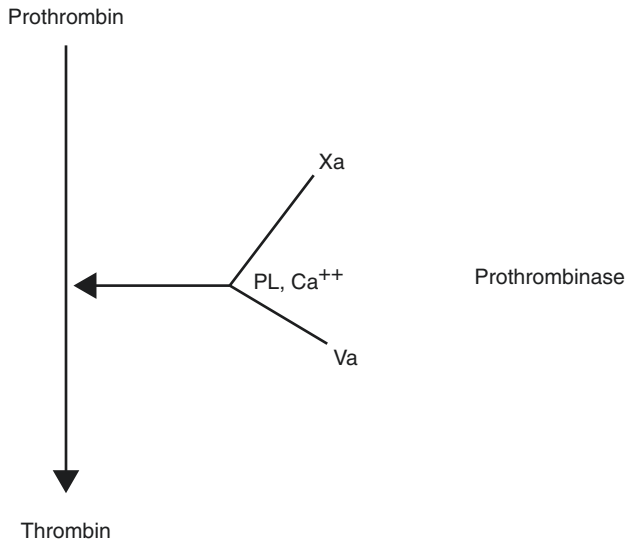

Fig. 10.2 The prothrombinase assembly for thrombin generation

2. *Prothrombinase assembly* (Fig. 10.2) is the ability of factor Xa and thrombin-activated factor Va to assemble on phospholipid membranes or cell membranes to accelerate the activation of factor II (prothrombin) to factor IIa (thrombin). When all of these components are present, the rate of factor II activation by factor Xa is increased 400,000-fold over the rate of factor Xa activation of factor II alone.
3. *Thrombin generation* in a kinetically fast manner is the goal of these protein assemblies. In static in vitro systems, as little as 5–10 pM (10^{-12} M) tissue factor is sufficient to induce clot formation leading to a 1000- to 4000-fold amplification of the process that increases the concentration of thrombin to 10–20 nM (10^{-9} M), a concentration sufficient to initiate clot formation. The addition of 5 pM tissue factor results in an average clot time of ~5 min, a time sufficiently fast for physiologic hemostasis.

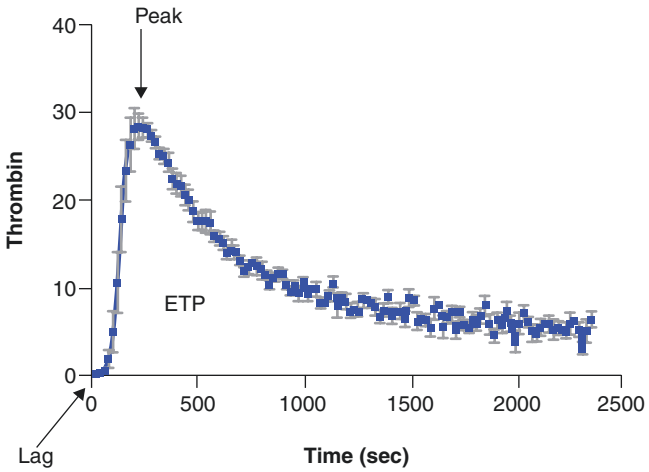

Fig. 10.3 Thrombin generation curve. "Lag" is the time from zero until there is an upward slope. "Peak" is the peak height of the generated thrombin curve. "ETP" stands for endogenous thrombin potential which is the total thrombin generated

Thrombin generation is shown graphically as a thrombin generation time (Fig. 10.3) [5]. Whether thrombin formation is initiated by tissue factor-factor VIIa or contact activation with factor XII, prekallikrein, and high-molecular-weight kininogen, the character of the thrombin generation time (TGT) curve is the same. In essence, the thrombin generation curve is a graphic prothrombin time or activated partial thromboplastin time (see Chap. 11) when it is induced by tissue factor-factor VIIa or a contact-activating surface, respectively. After an initial lag time (Lag) that varies due the potency of the stimulus, there is an acute increase in the formation of thrombin. The rate of rise (ΔTGT) and the peak height (Peak) of thrombin generation are measurable events. Further, the area under the curve (ETP – endogenous thrombin potential) directly correlates with the nM thrombin generated (Fig. 10.3). Thrombin generation curves represent the total amount of thrombin generated but do not indicate why thrombin generation is low (e.g., low factor VIII or high antithrombin levels). In general, it takes about 1 nM formed thrombin to activate platelets; 10 nM formed thrombin to generate a fibrin clot.

(C) *Summary of Physiologic Blood Coagulation System*

The assembly of blood coagulation proteins whose deficiency is associated with bleeding is shown in Fig. 10.4. Blood coagulation leading to hemostasis is initiated by the complex formation between tissue factor and factor VIIa. Tissue factor (TF) is expressed after injury upon exposure of subendothelium or is synthesized in monocytes, neutrophils, endothelium, or platelets in inflammatory states. It has been proposed that there may be TF-VIIa complexes in cryptic microparticles

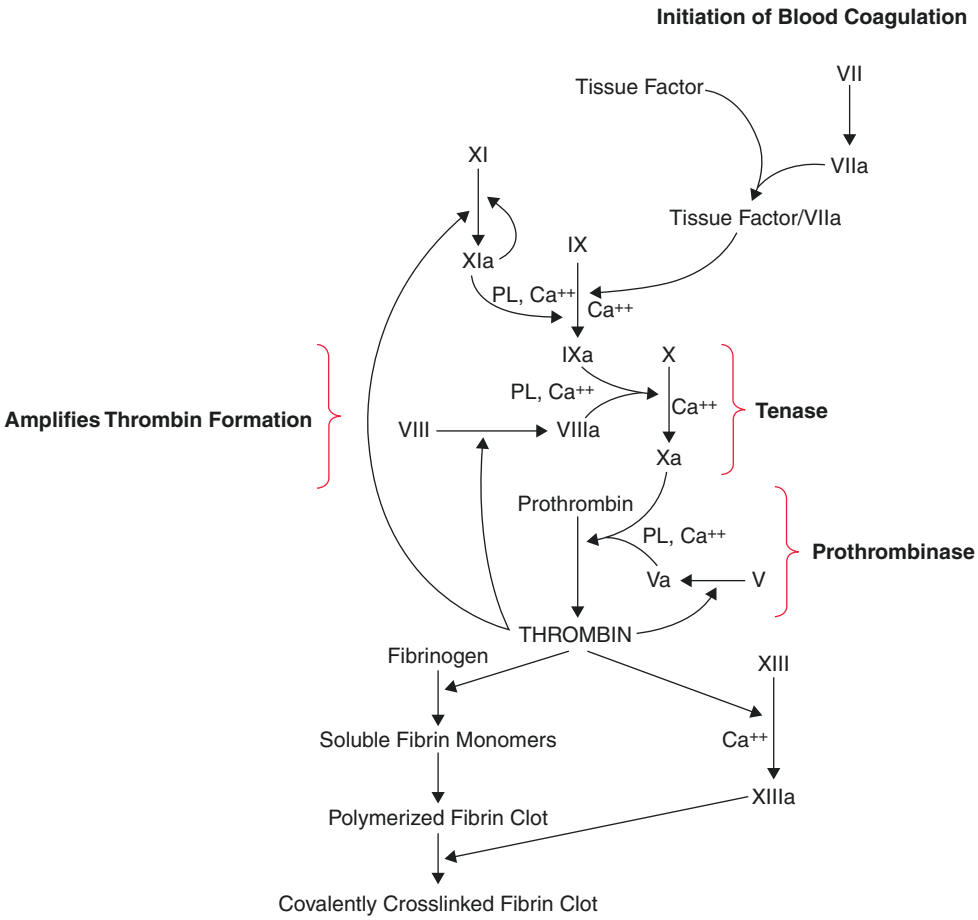

Fig. 10.4 Blood coagulation system leading to hemostasis

circulating in plasma that are available to spring into action if a hemostatic insult occurs. Although factor VIIa directly activates X to Xa in vitro in the prothrombin time assay (see Chap. 11), under physiologic conditions this pathway is blocked by a serine protease inhibitor called tissue factor pathway inhibitor (TFPI) (see below under anticoagulation systems). Physiologic blood coagulation mostly occurs when sufficient tissue factor/VIIa is available to activate factor IX to IXa. Subsequently, IXa in the presence of VIIIa assembles to activate X to Xa in tenase (Fig. 10.4). Formed Xa in the presence of Va leads to prothrombin activation to form thrombin in prothrombinase (Fig. 10.4). Formed thrombin proceeds to proteolyze (chew up) fibrinogen to form soluble fibrin monomers that polymerize to form a fibrin clot. Thrombin also activates factor XIII to XIIIa that cross-links polymerized fibrin monomers to form insoluble cross-linked fibrin, a clot. A clot, for example, is the soft bloody gel that hardens on a severely abraded knee. At times, the stimulus for thrombin formation can be great. Thrombin amplifies its own formation by feeding back to activate factor XI to factor XIa that activates more factor IX to reinitiate the cascade of proteolytic events just described. Please note there is no mention of the contact activation proteins, factor XII, prekallikrein, and high-molecular-weight kininogen in physiologic hemostasis. These proteins do not contribute to the cessation of bleeding. However, as already mentioned, these proteins can be activated to form clots when artificial surfaces are inserted into patients (e.g., cardiopulmonary bypass, extracorporeal membrane oxygenation, indwelling IV catheters) or in disease states like sepsis from any cause and adult respiratory distress syndrome.

The Fibrinolytic System

One process limiting the extent of clot formation is the *fibrinolytic protein system* (Fig. 10.5). It consists of the zymogen plasminogen and its naturally occurring activators. Plasminogen is activated to the main clot-lysing enzyme, plasmin, by the endogenous plasminogen activator tissue plasminogen activator (tPA), single-chain urokinase plasminogen activator (ScuPA), and two-chain urokinase plasminogen activator (tcuPA). These activators are found in the endothelium as well as in neutrophils and monocytes. Plasminogen activation is regulated by the inhibitor plasminogen activated inhibitor-1 (PAI-1). PAI-1 is mostly found in endothelium and cells; it is not a plasma protein. In inflammatory states, its production increases, and it is released into the circulation.

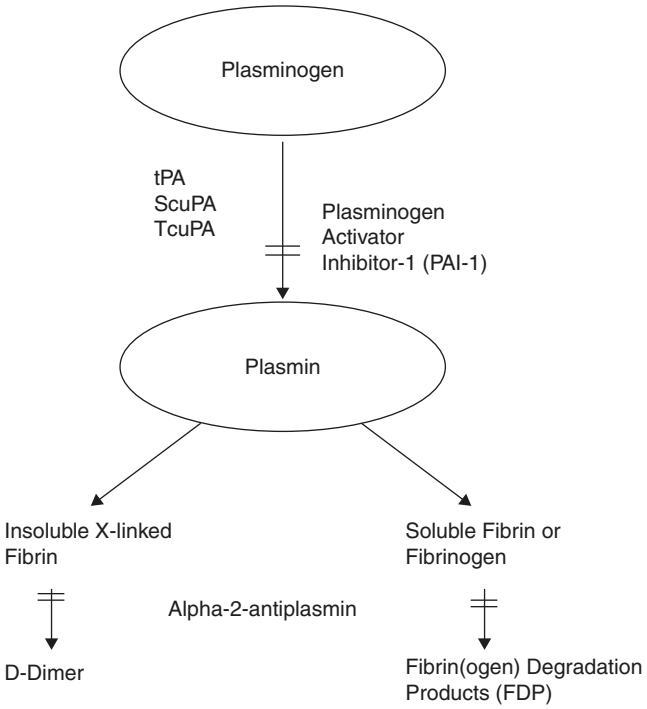

Fig. 10.5 The fibrinolytic protein system

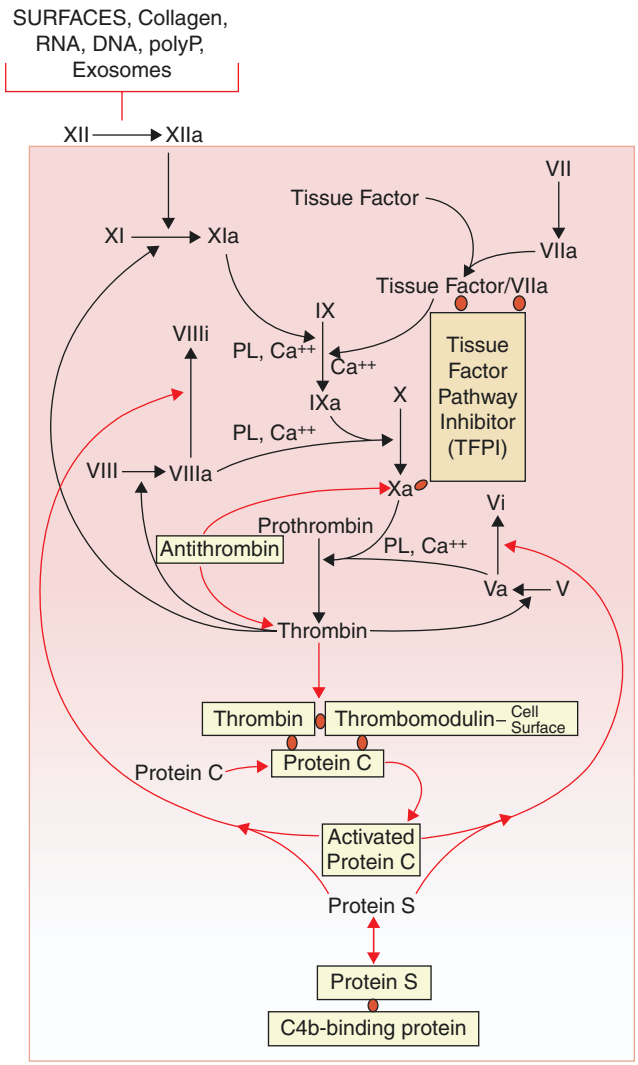

Fig. 10.6 The anticoagulation protein system. The anticoagulants are in the boxes in *yellow*

It is also found in platelets in large concentrations. Formed plasmin degrades fibrinogen, soluble non-cross-linked fibrin, and cross-linked fibrin to liberate fibrin or fibrinogen degradation products (Fig. 10.5). Plasmin degrades insoluble cross-linked fibrin clots to liberate D-dimer, i.e., a two D-domain protein fragments held together by a unique bond between them (Fig. 10.5). Measurement of D-dimer indicates that thrombin-activated factor XIII has cross-linked fibrin to make insoluble cross-linked fibrin and plasmin has cleaved the insoluble cross-linked fibrin. The plasma serine protease inhibitor, alpha-2-antiplasmin, regulates plasmin activity. A defect of plasminogen is associated with thrombosis. An absence or defect in PAI-1 or alpha-2-antiplasmin is associated with a hyperfibrinolytic (high rate of formed clot lysis) bleeding state. Elevations of PAI-1 or alpha-2-antiplasmin are associated with increased risk for myocardial infraction and stroke.

The Anticoagulation System

(A) A second process limiting the activation of the blood coagulation system is the anticoagulation systems. Three *anticoagulant systems* regulate activation of the *blood coagulation proteins* to inhibit clot formation. These systems are the protein C (PC)/protein S (PS) system, the plasma SERPIN serine protease inhibitor antithrombin (AT), and the Kunitz serine protease inhibitor tissue factor pathway inhibitor (TFPI). In Fig. 10.6, these anticoagulation systems with red lines are drawn over the blood coagulation system in black lines.

1. *Protein C/protein S system***.** When activated, *protein C* (PC), a vitamin K-dependent protein, is an enzyme that functions as an inhibitor. Protein C is activated to its enzymatic form by thrombin when bound in a trimolecular complex with an endothelial cell receptor called thrombomodulin. Activated protein C makes a complex with protein S to function as an inhibitor by degrading factor Va, a cofactor for prothrombinase, and factor VIIIa, a cofactor for tenase (Fig. 10.6). *Protein S* (PS), a vitamin K-dependent protein, is not an enzyme. It serves as a receptor for activated protein C to perform its activities on cell membranes. Protein S levels are modulated by the complement inflammatory protein C4b-binding

protein. Clinical deficiencies of protein C or S are associated with serious risk for thrombosis (see Chap. 16).

2. *Antithrombin anticoagulation system. Antithrombin* (AT), a serine protease inhibitor (SERPIN), exerts its anticoagulant effect primarily by inhibiting factors IIa and Xa (Fig. 10.6). It also inhibits each of the other hemostatic enzymes: factors VIIa, IXa, XIa, kallikrein, and XIIa (not shown in the figure). The presence of antithrombin is what gives heparin its anticoagulant properties. Heparin binds antithrombin and makes the latter a better inhibitor by changing the conformation of its reactive region. The importance of antithrombin is indicated by the fact that in the mouse, a complete deficiency results in fetal death. In the presence of heparin, antithrombin is a 1000-fold more effective inhibitor of factor IIa (thrombin). The clinical state of heterozygous deficiency of antithrombin is associated with venous thrombosis (see Chap. 16).
3. *Tissue factor pathway inhibitor (TFPI)*, a Kunitz-type serine protease inhibitor, is the third anticoagulation system. It is the most potent inhibitor of the factor VIIa/tissue complex. Under physiologic conditions, TFPI makes a quaternary complex with tissue factor, factor VIIa, and factor Xa to prevent the FVIIa-TF complex from activating factor X directly (Fig. 10.6). This fact directs FVIIa-TF to factor IX for activation. The importance of TFPI is indicated by the observation that in the mouse, a complete deficiency results in fetal death.

(B) *Additional Regulators (Anticoagulants) of Coagulation Proteins*

Additional serine protease inhibitors regulate some of the blood coagulation enzymes. However, the significance of their clinical deficiencies is still being defined. Heparin cofactor II is a thrombin inhibitor. Protein Z inhibitor in the presence of its cofactor protein Z is a factor Xa inhibitor. C1 esterase inhibitor (C1 inhibitor), alpha-1-antitrypsin, or the amyloid β-protein precursor are potent inhibitors of factor XIa. C1 inhibitor also is the major plasma protease inhibitor of factor XIIa and plasma kallikrein. Alpha-1-antitrypsin is the major inhibitor of elastase. The amyloid β-protein precursor also inhibits factors IXa, VIIa-tissue factor, and factor Xa, but not thrombin. It appears to be a cerebral anticoagulant, i.e., the major anticoagulant protein in the brain and also has an influence of venous thrombosis. C1 inhibitor deficiency is pathogenetic for the inflammatory disorder, Type I or Type hereditary angioedema. Alpha-1-antitrypsin deficiency is associated early emphysema and chronic obstructive pulmonary disease.

Cohesive Hypotheses for the Initiation of the Hemostatic System

It is challenging to have a cohesive understanding of the many parts contributing to normal hemostasis. Although there is an enormous understanding about the contributors to hemostasis, there is no global model, and several cogent mechanisms have been proposed, each of which may be relevant under different circumstances. In the present chapter, no detailed discussion is presented on the contribution of platelets to hemostasis (see Chaps. 14 and 15), an important additional contributor. Furthermore, physiologic hemostasis or thrombosis is not a linear sequence or cascade of enzymatic reactions. It is an event occurring in the intravascular compartment in the presence of flowing blood that produces shear forces, that is, an additional factor contributing to these events. Additionally, the initiation of hemostasis varies between arteries, a high-flow and -shear vessels, and veins, a low-flow and -shear tissue. With these caveats, a couple of models for in vivo hemostasis and thrombosis can be proposed. In the intravascular compartment, intact endothelium has a constitutive anticoagulant and antithrombotic nature by its secretion of nitric oxide, prostacyclin, and tissue plasminogen activator (tPA) and the presence on its membrane of antithrombin, thrombomodulin, and an ADP-degrading enzyme called ectoADPase (CD39). After injury or in disease states, the constitutive anticoagulant nature of the vessel wall can turn and be procoagulant.

In the platelet plug hypothesis that has been around for decades, at sites of developing injury, platelets under shear are slowed by adherence to von Willebrand factor (vWF) (see Chap. 12) and activated after their interaction with exposed collagen through the platelet receptor GPVI [1]. This initial platelet event at a site of vessel injury is called primary hemostasis. Exposed collagen, aggregated protein, cellular polyphosphates (polyP), or extracellular RNA also has the ability to bind plasma factor XII (XII) to support its autoactivation to factor XIIa (XIIa). Factor XIIa formed in such a manner leads to a cascade of activation of blood coagulation protein leading to thrombin formation. This pathway is not essential for hemostasis but may be important for pathologic thrombosis. Simultaneously or alternatively, injured subendothelium results in the expression of tissue factor which complexes with factor VII/VIIa to activate factor IX and leads to thrombin formation. This latter mechanism for thrombin formation is essential for hemostasis and is an alternative process for thrombosis. Formed thrombin by any mechanism leads to more fibrin clot and platelet activation through the platelet thrombin receptors, protease-activated receptors 1 and 4 (PAR1 and PAR4). These events additionally activate platelets and expose surface phospholipids like phosphatidylserine (PS) which itself is a

procoagulant, i.e., a thrombin-generating surface. Activated platelets also release ADP and serotonin to recruit more platelets and polyP, a lipid material that can support factor XII activation. Finally, most probably, different stimuli lead to different mechanisms for thrombin formation. These additional processes leading to thrombin formation are termed secondary hemostases.

In recent years, another understanding for the initiation of hemostasis has been demonstrated at least in the experimental model situation. For example, a laser injury to a small arterial blood vessel results in tissue factor, factor VIIa, and thrombin formation before platelets adhere and aggregate. The formed thrombin recruits platelets for a dense platelet thrombus, and additional platelet activation releases platelet ADP to recruit another layer of the platelet thrombus [6]. These secondary mechanisms lead to either vessel occlusion or thrombus dissolution depending upon the strength of the initial thrombin formation or secondary platelet response.

The complexity of these mechanisms is magnified by arteries versus veins, high shear versus low shear, large vessels versus small vessels, and healthy tissue versus atherosclerotic diseased vasculature. All of these processes are occurring variably simultaneously and contribute to bleeding cessation or hemostasis or unchecked occlusive thrombosis. Even though these systems are interacting, overlapping, and redundant, they function in an elegant balance. The remarkable thing is that the absence of only one factor alters the balance that leads to a bleeding or thrombotic state.

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Suggested Reading

General Reviews

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Approach to the Bleeding Patient

11

Alvin H. Schmaier

Introduction

This chapter aims to provide a simple diagnostic framework in which the physician can approach most patients with abnormal bleeding in a logical fashion to recognize the underlying cause. The diagnostic approach to bleeding disorders is based upon a full understanding of what the current screening assays for bleeding states measure.

Pathogenesis of Bleeding Disorders

When faced with a bleeding patient, the physician must use an analytical diagnostic approach to determine the etiology of the problem.

(A). *All bleeding states are caused by one of three defects*:
1. *Plasma protein defect* (i.e., a quantitative or functional defect in one or more plasma coagulation, fibrinolytic, or anticoagulant proteins)
2. *Platelet abnormality* (i.e., a quantitative deficiency or function defect in this hemostatic cell fragment – one of adherence, aggregation, spreading, and activation)
3. *Defect in platelet-endothelial cell interactions* (i.e., a defect in the adhesive interactions between platelets and the vessel wall)

(B). *Coagulation protein defects* that lead to bleeding are classified as follows:
1. *True protein deficiency*. Insufficient protein is present for its required function.
2. *Inhibition of an active region of a protein*. The protein is present, but an inhibitor to its function arises. These inhibitors are usually immunoglobulins, but other forms of inhibitors can be present as well (e.g., a tumor secreting a heparin-like substance).
3. *Production of abnormal protein molecule*. The protein is present, but as a result of a mutation, missense, or deletion, an active portion of the protein is altered such that it cannot participate in its physiologic functions.
4. *Enhanced clearance of the protein*. The antigen-antibody complex is recognized as foreign. An antibody arises against a normal protein and resulting in the complex being removed from the circulation. The resultant increased clearance of the protein gives the appearance of a deficiency.

Clinical Presentation of Bleeding Disorders

The clinical presentation of bleeding disorders is shown in Table 11.1.

Coagulation Cascade Hypothesis

This 50+-year-old theory of hemostasis still has merit as an approach to use the current, clinical laboratory screening tests (see below) for the differential diagnosis as to the nature of the defect. It is *not* a complete model of physiologic hemostasis (see Chap. 10). In the coagulation cascade

Table 11.1 Clinical presentation of bleeding disorders

	Hemophilioid state	Purpura
Bleeding source	Small artery	Capillary
Relation to trauma	Frequent	Rare
Presenting signs	Hematoma, ecchymosis	Ecchymosis, petechiae
Underlying cause	Factor deficiencies (e.g., VIII, IX, XI)	Platelet defects, von Willebrand disease
Bleeding time	Normal	Abnormal

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hypothesis, coagulation proteins are classified as members of the intrinsic system, the extrinsic system, or the common pathway (Fig. 11.1).

(A) *Intrinsic System*
1. Factor XII
2. Prekallikrein (PK)
3. High-molecular-weight kininogen (HK)
4. Factor XI
5. Factor VIII
6. Factor IX

(B) *Extrinsic System*
1. Factor VII
2. Tissue factor

(C) *Common Pathway*
1. Factor X
2. Factor V
3. Factor II
4. Factor I (fibrinogen)

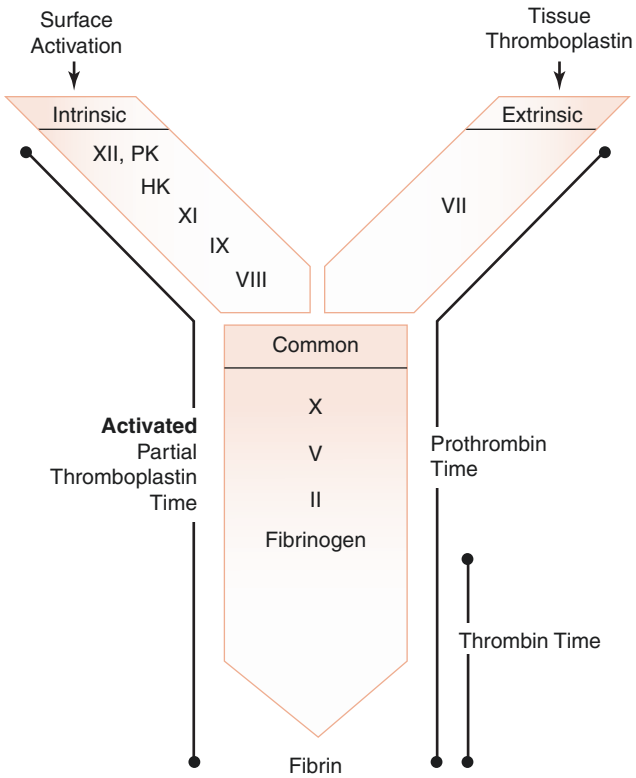

Fig. 11.1 Plasma coagulation assays and the factors they measure. The plasma proteins involved in in vitro coagulation reactions can be classified into three systems: intrinsic, extrinsic, or common. The screening tests for coagulation proteins [activated partial thromboplastin time (aPTT), prothrombin time (PT), or thrombin time (TT)] measure one or more of the proteins involved in in vitro clot formation. Knowing what test(s) is abnormal pinpoints the likely protein defect that will give this result. *HK* high-molecular-weight kininogen, *PK* prekallikrein

Screening Tests for Bleeding Disorders

Several screening tests are used to classify and diagnose bleeding disorders. When these assays are performed simultaneously on a sample of plasma, the results indicate almost all the diagnostic categories for a bleeding state.

(A) *Activated partial thromboplastin time (aPTT)*. To perform this coagulation assay, a mixture of a negatively charged surface, phospholipids and patient plasma are incubated for a few minutes (5–8 min). Plasma is made from whole blood anticoagulated with 3.2 g% sodium citrate (Chap. 1). With the addition of excess calcium chloride to overcome the plasma calcium chelation with citrate, the time required for clot formation in seconds is measured. The aPTT's assessment includes all the coagulation proteins of the intrinsic system and the common pathway. The only coagulation factor that does not influence the aPTT is factor VII.

(B) *Prothrombin time (PT)*. To perform this coagulation assay, a preparation of tissue factor (usually recombinant but can be isolated from animal tissues) is added to patient plasma and both are incubated for 5–8 min. Afterward, calcium chloride is added, and the time required for clot formation is measured. PT assesses the coagulation proteins of the extrinsic system and the common pathway.

(C) *Platelet count*. This assay measures the number of platelets in a microliter of blood. It is used to exclude a quantitative platelet defect as the cause of a bleeding disorder.

(D) *Bleeding time*. To perform this assay, the forearm is scratched using a special device, and the time until bleeding stops is measured. It assesses platelet number or function. It is not commonly used today because it is technically not reproducible and requires experienced individuals to perform the assay. Adequate practice of hemostasis does not require inclusion of the bleeding time assay.

(E) *Thrombin clotting time (TCT)*. To perform this assay, purified thrombin is added to plasma to determine the time for clot formation. It is a direct measure of fibrinogen amount and/or function only.

Interpretation of Screening Tests of the Proteins in the Coagulation System

Using the coagulation cascade hypothesis and its grouping of proteins, the screening tests for bleeding disorders can be used to measure specific coagulation proteins (Fig. 11.1).

(A). In the *aPTT*, factor XII autoactivates by exposure to an artificial negatively charged surface. The aPTT

measures the proteins of the intrinsic system (factor XII, prekallikrein, high-molecular-weight kininogen, factor XI, factor IX, and factor VIII) and the proteins of the common pathway (factors X, V, and II and fibrinogen). It does *not* measure factor VII.

(B). The *PT* measures the extrinsic system of the coagulation, which consists of activated factor VII (factor VIIa), and tissue factor which is part of the reagent for the assay and the proteins of the common pathway (factors X, V, and II and fibrinogen).

(C). *Thrombin time* only measures the ability of fibrinogen to clot after proteolysis by the addition of exogenous thrombin.

Differential Diagnosis of an Isolated Prolonged Activated Partial Thromboplastin Time

The following approach is used to evaluate patients who have an isolated prolonged aPTT:

(A). *Disorders associated with bleeding*

1. Congenital factor VIII deficiency or defect is sex-linked (i.e., carried on the X chromosome). Congenital factor VIII deficiency occurs only in males. Acquired factor VIII deficiency due to antibodies to the protein occurs in both males and females, usually elderly.
2. Factor IX deficiency or defect is sex-linked.
3. Factor XI deficiency is autosomal recessive.

(B). *Disorders not associated with bleeding*

1. Factor XII deficiency is autosomal recessive and is the most common type. It gives very prolonged aPTTs and is not associated with bleeding.
2. Prekallikrein (PK) deficiency is autosomal recessive. It gives a mildly prolonged aPTT that corrects on own if the plasma sits at room temperature on bench for 1 h. PK influences the rate of factor XII activation but is not essential for factor XII autoactivation.
3. High-molecular-weight kininogen (HK) deficiency is autosomal recessive and extremely rare. It gives a very prolonged aPTT. HK serves as a cofactor for factor XII, prekallikrein, and factor XI activities.
4. Lupus anticoagulants are antiphospholipid antibodies that interfere with coagulation reactions. These antibodies arise for many reasons and are not specific to illness, although they are often seen in patients who have connective tissue disorders [e.g., systemic lupus erythematosus (42%)]. Usually, they do not interfere with the protein itself but rather with the phospholipids reagents that are used in the coagulation assay. Thus, calling these entities "anticoagulants" is a misnomer. Although these antibodies prolong coagulation protein assays, they are not associated with bleeding unless hypoprothrombinemia or thrombocytopenia is present. Paradoxically, they are often associated with thrombosis because they interfere with endothelial cell-based anticoagulation systems. When lupus anticoagulants prolong the aPTT, it is a diagnosis made by exclusion of other specific entities that are associated with bleeding (e.g., FVIII, FIX, and FXI deficiency). It is important to note that a lupus anticoagulant does not only prolong the aPTT, it can at times prolong the PT. The diagnosis of a lupus anticoagulant is made by assays designed to detect an entity interfering with the assay's reagents and not coagulation factors. Currently, assays such as the dilute Russell viper venom time (DRVVT), tissue thromboplastin inhibition assay (TTI), dilute aPTT, and kaolin or silicon clotting time are used. All these assays reduce the concentration of the reagent in the assay so that in the presence of the inhibitor, the defect is amplified in relation to a normal sample run simultaneously. Additionally, one examines for antibodies to phospholipids such as anticardiolipin and anti-beta-2-glycoprotein I. If antibody titers are positive twice over 3 months, this observation is consistent with diagnosis of antiphospholipid antibody syndrome.

Differential Diagnosis of a Prolonged Prothrombin Time Only

An isolated PT prolongation usually indicates factor VII deficiency. Most of these patients usually have partial VII defects. Occasionally, isolated prolonged PT is seen in a patient who has dysfibrinogenemia (abnormal and/or deficient fibrinogen) or a deficiency in coagulation factors X, V, or II. These results are dependent upon the sensitivity of the tissue factor (thromboplastin) used in the clinical of reagents a clinical laboratory employs. In general, recombinant human tissue factor is the most sensitive reagent.

Differential Diagnosis of a Prolonged Activated Partial Thromboplastin Time and Prothrombin Time

Usually, these abnormalities in the coagulation protein screening tests are not specific protein conditions but rather acquired general medical conditions as seen in acquired bleeding disorders (see Chap. 13). These abnormalities may be caused by many reasons.

(A). Medical causes include disseminated intravascular coagulation (see Chap. 13), liver disease, vitamin K deficiency, the use of therapeutic anticoagulants (e.g., heparin, warfarin, low-molecular-weight heparin, fondaparinux, argatroban, bivalirudin, dabigatran, rivaroxaban, apixaban, edoxaban, betrixaban), and massive transfusion with an anticoagulation effect from receiving too much acid-citrate-dextrose anticoagulant in the infused blood products.

(B). Hypofibrinogenemia/dysfibrinogenemia occurs when a low and/or abnormal fibrinogen molecule does not participate properly in coagulation reactions. A normal blood coagulation time requires a plasma fibrinogen to be ≥100 mg/dl.

(C). In rare cases, patients with low factors X, V, or II are seen, and these patients will present with a prolonged aPTT and PT.

Use of the Activated Partial Thromboplastin Time and Prothrombin Time to Monitor Anticoagulation

1. *aPTT*. The aPTT is often used to monitor the extent of anticoagulant produced by unfractionated heparin, argatroban, and bivalirudin. Use of the aPTT to monitor these anticoagulants is expedient but not ideal. The aPTT should only be considered a general guide on the degree of anticoagulation in patients. The increase in the aPTT in a patient on unfractionated heparin, argatroban, or bivalirudin is not linearly related to the concentration of the anticoagulant. The aPTT will prolong in a hyperbolic, not linear manner, as the anticoagulant concentration is used. Direct measurement of unfractionated heparin levels is determined by neutralization of factor Xa or IIa activity. Direct measurement of argatroban or bivalirudin levels, two agents that are direct thrombin inhibitors, is determined by neutralization of factor IIa activity. The aPTT is best as a diagnostic test to determine if there is specific protein defect of the intrinsic system. Its prolongation alone does not mean there is risk for bleeding since long aPTTs are associated with factor XII, prekallikrein, high-molecular-weight kininogen deficiencies, and lupus anticoagulants, all of which are not bleeding states.
2. *PT*. The prothrombin time is used to monitor the oral anticoagulant warfarin. When using the PT to monitor warfarin, the degree of anticoagulation is expressed as a value called the INR – international normalized ratio. The INR is determined by the ratio of the patient's PT to the mean PT performed in the laboratory on a population of normals ($n > 20$). If a patient sample is normal, then the INR is ~1.0. The therapeutic levels of warfarin usually have an INR between 2 and 3.This value is about 20% normal functional levels of the vitamin K-dependent coagulation factors II, VII, and X and 40% normal function of factor IX.

Differential Diagnosis of a Prolonged Bleeding Time and a Normal Platelet Count

Defects in bleeding time in the presence of a normal platelet count signify abnormalities in the following:

(A). *Von Willebrand factor* (von Willebrand disease) (see Chap. 12)

(B). *Platelet function* (see Chaps. 13 and 14)

(C). *Rare connective tissue disorders*, including pseudoxanthoma elasticum, Ehlers-Danlos syndrome, and scurvy that is associated with collagen breakdown and perifollicular hemorrhage

Prolonged Bleeding Time as a Result of Platelet Defects

This abnormality occurs when the number of platelets is decreased or when a true intrinsic defect in platelet function occurs.

(A). *Quantitative decrease in platelet count*. As the platelet count decreases to less than 100,000/μl, the bleeding time becomes prolonged.

(B). *True platelet function defect* (see Chaps. 13 and 14).

Differential Diagnosis of a Bleeding State that Is Not Associated with an Abnormality in the Screening Tests

These entities are rare and mainly consist of abnormalities of the fibrinolytic system (e.g., defects in alpha-2-antiplasmin or plasminogen activator inhibitor) or in factor XIII.

(A). Factor XIII deficiency may be congenital or acquired. Factor XIII is a thrombin-activated transglutaminase that crosslinks fibrin monomers into polymers that produces the fibrin clot. Patients bleed excessively as a result of surgery or trauma. Middle-aged adults have a high incidence of spontaneous intracerebral hemorrhage.

(B). Alpha-2-antiplasmin deficiency is the absence of the major serine protease inhibitor (SERPIN) of plasmin.

These patients have a bleeding disorder that is caused by a hyperfibrinolytic state, allowing for lyses of any clots that are formed. Alpha-2-antiplasmin deficiency can be acquired as result of consumption in disseminated intravascular coagulation (e.g., acute leukemia especially acute promyelocytic anemia).

(C). Plasminogen activator inhibitor-1 deficiency is a deficiency in the major serpin inhibitor of plasminogen activators. This abnormality causes increased activation of plasminogen. A pro- or hyperfibrinolytic state like alpha-2-antiplasmin deficiency also occurs as a result.

(D). Alpha-1-antitrypsin $_{\text{PITTSBURGH}}$ is an abnormal serpin that changed alpha-1-antitrypsin into a very potent antithrombin. It causes a severe bleeding disorder because it has exceedingly tight binding and potent inhibition of the activity of thrombin. Thus, any thrombin that forms is rapidly neutralized, and as a result, no clotting can occur, and the patient hemorrhages. This entity was recognized in a little girl with a lifelong bleeding defect leading to her demise at the age of 7.

Summary

The physician can recognize the underlying cause of most bleeding disorders that one may encounter in clinical practice by having a clear understanding what the aPTT, PT, platelet count, and thrombin time measure. It is of great clinical value to fully understand the results of these assays for the diagnosis and management of potential hemostatic disorders.

Suggested Reading

Napolitano M, Schmaier AH, Kessler CM. Coagulation and fibrinolysis. In: McPherson P, editor. Henry's clinical diagnosis and management by laboratory methods. 23rd ed. Philadelphia: Elsevier; 2017. p. 794–811.

Schmaier AH. Laboratory evaluation of hemostatic and thrombotic disorders. In: Hoffman R, Benz EJ, Silberstein LE, Heslop H, Weitz JI, Anastasi J, editors. Hematology: basic principles and practice. 6th ed. Philadelphia: Elsevier; 2013. p. 1857–66.

Congenital Bleeding Disorders

12

Rohith Jesudas and Steven W. Pipe

Overview

- The clinical phenotype of congenital bleeding disorders ranges widely dependent upon the type and severity of factor deficiency.
- Laboratory diagnosis of a coagulation factor deficiency is guided by the results of screening tests, including prothrombin time (PT), activated partial thromboplastin time (aPTT), and thrombin time (TT), and confirmed with specific factor assays.
- While specific factor replacement therapy, prophylactically where appropriate, is the foundation of clinical care for these disorders, non-replacement therapies may be alternative options for treatment.

Hemophilia

- Hemophilia is the most common inherited bleeding disorder. Conventionally, hemophilia refers to deficiencies of the coagulation proteins, factor VIII (FVIII), and factor IX (FIX).
- FVIII deficiency is known as hemophilia A or classic hemophilia.
- FIX deficiency is known as hemophilia B or Christmas disease. Historically, hemophilia was known as the royal disease as it affected members of Queen Victoria's family, beginning from her as the carrier. It has been confirmed that this was hemophilia B and Victoria likely acquired a spontaneous mutation in the *F9* gene that her two daughters and son inherited. The disease impacted Russian history as the Romanovs, in seeking treatment for the hemophilia affecting Victoria's great-grandson Prince Alexei, gave the Russian mystic Gregory Rasputin significant influence within the royal family, which some historians claim contributed to the fall of the Romanov empire.

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Role of FVIII and FIX in Hemostasis

- Factor VIII is synthesized by the endothelial cells in the lung and liver, while FIX is synthesized in hepatocytes. They participate in the intrinsic pathway of blood coagulation. The primary function of the intrinsic pathway is to amplify thrombin generation and facilitate formation of a strong fibrin clot at the site of vascular injury to arrest bleeding (See Chap. 9).
- FVIII circulates in plasma non-covalently bound to its carrier protein, von Willebrand factor (vWF). Upon activation by thrombin, it dissociates from vWF and acts as a cofactor for FIXa to activate factor X (FX) to FXa. The assembly of FVIIIa, FIXa, and FX on the phospholipid surface constitutes the intrinsic "tenase" complex. FXa generated from the intrinsic tenase complex is critical for amplifying the conversion of prothrombin to thrombin in the presence of its cofactor, factor Va, within the "prothrombinase" complex (See Chap. 9).
- Deficiency of FVIII or FIX leads to significant reduction in thrombin generation resulting in reduced fibrin clot formation that manifests as severe bleeding after even minor trauma and an increased tendency to re-bleed during physiologic clot lysis [1].

Epidemiology

- *Incidence*: The incidence of hemophilia A is approximately 1 in 5000 live male births, while the incidence of hemophilia B is 1 in 30,000 live male births.
- *Race*: Hemophilia has no racial predilection and is present in all ethnic and racial groups.

H. M. Lazarus, A. H. Schmaier (eds.), *Concise Guide to Hematology*, https://doi.org/10.1007/978-3-319-97873-4_12

- *Family history*: Although the majority (~70%) of cases of hemophilia are inherited, ~30% of cases arise from a spontaneous mutation, with no family history of hemophilia.

Genetics

- *Genetic locus*: The genes for both factor VIII (*F8*) and factor IX (*F9*) are located near the terminus of the long arm of the X chromosome, Xq28 and Xq27.1, respectively. *F8* gene is 186 Kb and consists of 26 exons, while *F9* spans 33 Kb and contains 8 exons.
- *Relationship between mutation type and the disease phenotype*: The type of mutations within the *F8* or *F9* gene predicts the disease severity. Typically deletions, insertions, and nonsense mutations result in severe disease, while missense mutations are more often found in mild to moderate disease.
- *Commonly encountered mutations*:
  - Most patients with hemophilia A have severe disease, and 40% of patients with severe hemophilia A carry an intron 22 gene inversion. This condition is caused by an intrachromosomal recombination between a 9.5 kb sequence within intron 22 of the *F8* gene and 1 of 2 closely related inversely oriented sequences located 5′ to the *F8* gene. An additional inversion within intron 1 of the *F8* gene is found in 5% of patients with severe hemophilia A. Most reference laboratories carry out initial screening for intron 22 inversion in cases of severe hemophilia A.
  - Most patients with hemophilia B have mild disease with missense mutations most common. Up to 25% of Caucasian patients have 1 of 3 founder mutations, (Gly106Ser, Thr342Met, and Ile443Thr). These are mutations thought to have been introduced into a population by an individual that was then passed on to subsequent generations and maintained within the gene pool [3].
- *Mode of inheritance*: Hemophilia A and B are transmitted as X-linked recessive disorders; hence males are typically affected, and females are carriers of the disease. All the daughters of an affected male are "obligate carriers" of hemophilia, while all his sons are unaffected. A carrier female has a 50% risk of transmitting her affected X chromosome with each pregnancy; therefore, with each male fetus, there is a 50% chance of being affected with hemophilia, and with each female fetus, there is a 50% chance of being a carrier.
- *Universal genotyping for patients and carriers of hemophilia:* Recently, there has been an effort to identify the genetic abnormalities in the hemophilia genes in all patients. Identifying all mutations and describing the phenotype will improve our efforts to offer individualized treatment options for patients with hemophilia.

Clinical Classification

Hemophilia is classified as mild, moderate, and severe on the basis of the patient's residual FVIII and FIX blood concentrations (1 IU/dL = 1%).

- Severe hemophilia: <1%
- Moderate hemophilia: ≥1–5%
- Mild hemophilia: ≥6–40%

Bleeding Manifestations

Overview

The hallmark of hemophilia is hemarthroses (joint bleeds), but bleeding manifestations may include any of the following: prolonged bleeding post-circumcision, neonatal intracranial bleeding or cephalohematomas, muscle hematomas, prolonged bleeding/oozing or renewed bleeding after trauma or surgical procedures, unexplained gastrointestinal bleeding or hematuria, recurrent epistaxis, and excessive bruising.

- Bleeding manifestations vary depending on the severity of the factor deficiency, type of hemophilia, and the age at presentation. Patients with severe hemophilia may experience spontaneous bleeding, while patients with moderate hemophilia bleed after trivial trauma. Without prophylactic therapy, patients with severe hemophilia typically experience 4–6 bleeding events per month or 20–30 events/year, while patients with moderate hemophilia typically experience 4–6 bleeding events per year. Clinical spectrum ranges from musculoskeletal bleeding such as hemarthrosis and muscle bleeding to, rarely, bleeding within internal organs.
- Bleeding in mild hemophilia may only manifest with significant trauma or with surgery. Bleeding pattern is predominated by mucocutaneous bleeding such as bruising, epistaxis, and prolonged bleeding after minor trauma.
- In general, patients with hemophilia B have relatively milder bleeding tendency compared to hemophilia A for the same residual factor levels.
- The clinical symptoms of bleeding in female carriers of hemophilia depend upon the baseline factor levels. Although the majority are asymptomatic, some female carriers of hemophilia A and B have significant reduction of their FVIII and FIX through skewed lyonization of the normal X chromosome. Lyonization is a process by which one of the two copies of the X chromosomes in females are inactivated so that only genes from the other active chromosome are expressed.

- Though typically random, disproportionate inactivation of the normal X chromosome within the expressing cells of female carriers with hemophilia can result in significantly reduced production of FVIII and FIX.
- A woman with a clotting factor of <40% should be treated the same as a man with similar levels (she has hemophilia). Women with factor levels >40% and who show bleeding symptoms are now classified as symptomatic carriers. Specifically, symptoms of menorrhagia and postpartum hemorrhage are common.

Hemarthrosis and Hemophilic Arthropathy

- Hemarthrosis, bleeding within the joint space, occurs spontaneously or after minor trauma. The frequency and age of onset of joint bleeding depends upon the severity of deficiency. In severe deficiency, joint bleeds typically begin between age 6 months and 6 years and can occur several times a month.
- Repeated bleeding (four bleeds into the same joint) in a 6-month period is considered a "target joint." The repeated bleeding initiates synovitis and ultimately progression to hemophilic arthropathy.
- Hemophilic arthropathy typically has five stages:
  - Stage I: Soft tissue swelling and inflammation due to bleeding within and around the joint
  - Stage II: Osteoporosis and epiphyseal overgrowth with an intact joint space and no bone cysts
  - Stage III: Subchondral cyst formation and joint surface irregularities with preservation of joint space
  - Stage IV: Subchondral cyst formation, prominent joint surface irregularities, and narrowing of joint space due to cartilage destruction
  - Stage V: Loss of joint space and epiphyseal overgrowth
- *Treatment:* Progression of hemophilic arthropathy leads to loss of range of motion and joint function. Prophylaxis treatment regimens prevent the progression of hemophilic arthropathy by reducing the frequency of joint bleeds, but do not necessarily reverse the joint damage. Surgical or radionuclide synovectomy and in some cases synovial embolization can be performed to reduce the bleeding tendency. Joint replacement surgeries are frequently required in adult hemophilia patients.

Muscle and Soft Tissue Bleeds

- Muscle bleeding is the second most frequent type of bleeding in patients with hemophilia. Intramuscular hemorrhages within a closed compartment such as the volar aspect of the wrist, deep palmer compartments of the hand, anterior or posterior tibial compartments, and inguinal region cause significant morbidity due to compression of neurovascular bundles (compartment syndrome).
- Treatment: Besides treatment with factor concentrates (detailed below) to raise the factor levels to the hemostatic range (>30–50%), immediate surgical decompression may be necessary to release the pressure and prevent irreversible tissue damage (e.g., Volkmann's contracture due to compartment syndrome within the forearm).

Life-Threatening Bleeds

Bleeding into areas that can compress vital structures (e.g., central nervous system, upper airway) or that can lead to excessive blood loss (e.g., gastrointestinal or iliopsoas hemorrhage) can be life-threatening and require prompt aggressive treatment with factor concentrates and possibly surgical management (Table 12.1).

Table 12.1 Characteristics of hemophilia and its management

	Hemophilia A		Hemophilia B	
Underlying deficiency	Factor VIII deficiency		Factor IX deficiency	
Subtypes according to severity	Severe, <1%; moderate, 1–5%; mild, ≥6–40%		Severe, <1%; moderate, 1–5% mild, ≥6–40%	
Therapy considerations				
Dose increment	A unit of exogenous FVIII will increase in vivo plasma FVIII levels by 2%		A unit of exogenous FIX will increase in vivo plasma FVIII levels by 1%	
Dose calculation	Units required to raise blood levels (%) = 0.5 Units/kg × body weight (Kg) × desired increase (%)		Units required to raise blood levels (%) = 1 Unit/kg × body weight (Kg) × desired increase (%)	
Mode of administration	Intravenous bolus or continuous infusion		Intravenous bolus or continuous infusion	
	Standard factor products	Extended half-life	Standard factor products	Extended half-life
Half-life	8–12 h	1.2- to 1.5-fold extension	~18 h	three to fivefold extension
Prophylaxis therapy[a]	25–40 IU/kg, q 3–4 times/week	40–50 IU/kg, q 3–5 days	40–60 IU/kg 2×/week	50–100 IU/kg 7–14 days
Recommended dosing for acute bleeds or surgical interventions				
Mild to moderate bleeding events (joint bleeding, cuts, minor trauma, minor surgery, soft tissue bleeding)	30–50% correction × 1–7 days		30–50% correction × 1–7 days	
Major bleeding events (CNS bleeding, any life-threatening bleeding)	100% correction until bleeding is controlled and 50–100% correction × 1–3 weeks followed by prophylaxis		100% correction until bleeding is controlled	
			50–100% correction × 1–3 weeks followed by prophylaxis	

[a]Dosage and frequency of administration may vary between the individual products and patients

Diagnosis of Hemophilia

The diagnosis of hemophilia is suspected based on personal history of the typical clinical phenotype of bleeding and family history of bleeding. History of hemarthrosis and the X-linked inheritance pattern are important clues for the diagnosis of hemophilia. Special coagulation tests are mandatory to confirm the diagnosis.

Special Coagulation Tests

- *Activated partial thromboplastin time (aPTT)*: Deficiency of FVIII and FIX prolongs the aPTT. In severe hemophilia, the aPTT is usually two to three times the upper limits of normal. In mild hemophilia, the aPTT assay can be normal. Therefore, in patients with a suspected bleeding disorder, specific factor assays should be performed to rule out FVIII or FIX deficiency. Patients with deficiencies of the contact pathway (factor XII, prekallikrein, and high molecular weight kininogen) also have significant prolongation of the aPTT without clinical symptoms of bleeding; hence, it is essential to be precise in the reason for the prolonged aPTT (see Chap. 11).
- *Chromogenic factor assay:* Most labs perform a one-stage assay to evaluate factor concentration. Should there be a discrepancy between the clinical picture and the factor level, consider evaluating factor levels with a chromogenic factor assay. This test is not routinely available in all centers.
- *Other screening tests of hemostasis*: Platelet count, prothrombin time, and thrombin time are normal in patients with hemophilia.

Genetic Testing

- Genetic testing for families with hemophilia is rapidly becoming part of routine clinical care.
- Testing for the commonly encountered mutations such as inversion 22 and inversion 1 is the recommended first step in evaluating a patient with severe hemophilia A. In the absence of commonly encountered mutation, next-generation sequencing of the *F8* and *F9* genes to identify the mutations within these genes may be performed.
- Identification of a mutation in a proband is particularly useful in carrier detection and prenatal diagnosis of hemophilia.
- In cases with borderline low factor VIII/IX levels (~40% to 50%), it is often prudent to confirm the diagnosis of mild hemophilia by performing genetic mutation analysis.

Special Considerations in Newborns

- Neither factor VIII nor factor IX crosses the placenta; thus, these patients can present with bleeding symptoms during the neonatal period.
- *Clinical symptoms*: Majority of bleeding symptoms in the newborn period are related either to birth trauma (e.g., cephalohematoma or intracranial hemorrhage) or interventions and procedures, such as injections, heel stick, and circumcision.
- *Laboratory diagnosis*: Specific factor assays are required to confirm the diagnosis of hemophilia since the aPTT assay may be prolonged in the newborn period due to deficiency of vitamin K-dependent proteins.
  - *Hemophilia A*: FVIII concentrations are at adult levels in the newborns; therefore, diagnosis of severe and moderate hemophilia A can be made in the newborn period. However, the diagnosis of mild hemophilia can be challenging as the FVIII levels are often increased in the newborn period in response to acute stress and maternal hormones. Repeat testing after several months is recommended.
  - *Hemophilia B*: FIX is a vitamin K-dependent protein, and the level of FIX could be physiologically low in the newborn period. Therefore, the diagnosis of mild and moderate hemophilia B needs to be confirmed after the newborn period.

Prenatal Testing

Prenatal diagnosis of hemophilia in a male fetus is offered to carrier females to provide additional information to guide pregnancy and delivery management. This is performed by preimplantation diagnosis, chorionic villous sampling at 10–12 weeks of gestation, or amniocentesis at 15–16 weeks of gestation. These procedures are however associated with risks to fetus and mother. Preimplantation genetic diagnosis is complementary to in vitro fertilization, which may be associated with the complications related to that procedure. Chorionic villus sampling and amniocentesis are associated with risk of bleeding (to both mother and fetus) and miscarriage. Recently, noninvasive prenatal testing of fetal DNA found in the maternal plasma has been shown in research settings to identify the fetal hemophilia status. If this becomes available as a clinical test, it will improve the perinatal management of at risk infants, while avoiding the potential morbidity associated with invasive testing.

Principles of Management of Hemophilia

Factor Replacement Therapies [5]

- Replacement of deficient coagulation factors by administration of exogenous factor concentrates is the primary treatment for patients with hemophilia. These clotting factor concentrates are either derived from plasma fractionation or recombinant technology.

The commonly used treatment regimens fall into one of five categories:

1. *Primary prophylaxis*: The regular and long-term administration of clotting factor concentrates to prevent bleeding

is the current standard of care. This has been shown to prevent recurrent joint bleeds and preserve joint function into adulthood. It is typically started before the age of 2 years and after no more than one joint bleed.
2. *Secondary prophylaxis*: Regular continuous (long-term) treatment started after two or more large joint bleeds but before the onset of joint disease.
3. *Tertiary prophylaxis*: Regular continuous treatment started after the onset of joint disease to prevent further joint damage.
4. *Intermittent prophylaxis*: Treatment is given to prevent bleeding for a short period of time, such as during and after surgery.
5. *Episodic (on demand) treatment*: Treatment is given at the time of bleeding.

The choice of treatment regimen depends upon:

- Severity of hemophilia
- Frequency of bleeding
- Severity of the bleeding event

Primary prophylaxis (with standard factor replacement products), typically 25–50 IU/kg administered three times per week to every other day, is now the standard of care for patients with severe hemophilia A and twice a week for severe hemophilia B. This has been demonstrated to prevent joint bleeds and subsequent arthropathy.

- Modified recombinant factor products, which extend the half-life in the plasma, are now available for treatment of hemophilia A and B. These modifications increase the half-life of FVIII 1.2- to 1.5-fold and 3- to 5-fold for FIX. This extension of half-life is achieved by linking the factor to albumin, the Fc fragment of immunoglobulin G, or addition of polyethylene glycol conjugates. A recombinant single-chain factor VIII product and a recombinant factor VIII molecule expressed in human cell lines have demonstrated improved interaction of FVIII with its carrier protein, vWF. This may also contribute to prolong the half-life of recombinant FVIII (Table 12.2) [2, 8].

Table 12.2 Extended half-life recombinant factor products available for patients with hemophilia

Modified recombinant agent	Description of the modification	Mechanism of half-life extension	Status
rFVIIIFc rFIXFc	Fusion of Fc domain of immunoglobulin G1 to recombinant FVIII and FIX	Neonatal Fc receptor (FcRn) on the cell surface binds to the Fc domain. This diverts the Fc fusion protein from lysosomal degradation and extends survival	Approved for clinical use
PEGylated FVIII	Covalent attachment of polyethylene glycol to recombinant factor	PEGylation reduces the binding of the PEGylated protein to its clearance receptors	Approved for clinical use
rFIX-FP	Genetically fused recombinant FIX and recombinant albumin	FcRn mediated recycling of albumin and its bound recombinant protein	Approved for clinical use
rVIII-single chain	Single-chain recombinant FVIII	Improved interaction with vWF, the FVIII carrier protein by optimizing structure and tyrosine sulfation	Approved for clinical use
Human-cl rhFVIII	Factor expressed in human cell lines	Improved interaction with vWF which is the FVIII carrier protein by optimizing structure and tyrosine sulfation and elimination of the nonhuman glycans	Approved for clinical use

Substitution and Hemostatic Rebalancing Therapies

- More recently a substitution therapy has been developed that may revolutionize the treatment of hemophilia. The first product to be commercially available is emicizumab. This is a humanized bispecific monoclonal antibody that seeks to imitate the cofactor function of FVIIIa by recognizing and bringing together FIX/FIXa and FX/FXa to facilitate generation of FXa. It has no structural homology to FVIII; therefore, it would not be expected to induce FVIII inhibitors or be affected by the presence of FVIII inhibitors. It has a high bioavailability administered subcutaneously and a long half-life of ~30 days. It is currently approved in several countries for once-weekly prophylaxis in persons with hemophilia A with inhibitors of all ages. However, clinical trial results have shown a high level of safety and efficacy in hemophilia A, with and without inhibitors, administered up to a 4-week dosing regimen.
- Hemostasis is maintained in an equilibrium to ensure that blood flows freely but can still clot when necessary. This equilibrium is maintained by procoagulants (e.g., factors VIII and IX) balanced with natural anticoagulants (tissue factor pathway inhibitor, TFPI; antithrombin; proteins C and S). When there is a deficiency of a clotting factor, hemostatic rebalancing has typically been provided through factor replacement therapy (or now recently through substitution therapy). However, it has been observed that patients with hemophilia, who have co-inherited thrombophilic risk

factors, can have a milder bleeding phenotype. In this manner, hemostatic rebalancing occurs through reduction in the influence of the natural anticoagulants. There are now clinical research programs investigating the targeting of each of the key natural anticoagulant pathways. Antithrombin can be targeted by a small interfering RNA (siRNA) which leads to posttranscriptional inhibition of antithrombin production (Table 12.3). Also in development are anti-TFPI antibodies which inhibit the action of TFPI. TFPI regulates the initiation of thrombin generation by the inhibition of tissue factor-factor VIIa complex. Most recently an engineered serine protease inhibitor, based on alpha-1-antitrypsin, has demonstrated specificity for inhibition of activated protein C. In each of these latter examples, inhibition of the natural anticoagulant pathway leads to more robust thrombin formation without replacing the deficient clotting factor (FVIII or FIX). Such therapies would be expected to application to patients with and without inhibitors to factors VIII and IX and could even have efficacy in other rare bleeding disorders [4].

- *Recommended Treatment for Acute Bleeding*
- In the event of an acute bleeding episode, it is critical to raise the levels of FVIII or FIX to the hemostatic range. The intensity and duration of factor replacement therapy depend upon the severity of the bleeding (Table 12.1).
  - *Minor bleeding episodes*: It is recommended to raise the factor level up to at least 30–50%. Usually one or two doses are enough to control minor bleeding events.
  - *Major bleeding episodes* (e.g., *CNS bleeds*): factor levels should be raised up to 80–100% until the bleeding is arrested. Subsequent maintenance of hemostatic levels will depend upon the severity of bleeding episode and its response to treatment and may be achieved by continued bolus injections or a continuous infusion. Table 12.1 illustrates the recommended dosing regimens for commonly encountered bleeding manifestations in patients with hemophilia.

Adjuvant Therapies

Desmopressin (DDAVP) is a synthetic peptide that causes release of vWF from storage sites within endothelial cells. This agent also results in a parallel increase in plasma FVIII levels. DDAVP is particularly useful in patients with mild hemophilia A and may obviate the need for factor replacement therapy with minor bleeds such as epistaxis or gum bleeding. Other adjuvant therapies such as antifibrinolytic medications (aminocaproic acid and tranexamic acid) and topical hemostatic agents (thrombin, fibrin sealant) may also be used even in severe hemophilia along with specific factor replacement therapy.

Inhibitors of Coagulation Factor VIII and Factor IX

- *Definition*: Alloantibodies developed against exogenously administered factor (FVIII or FIX) that interfere with their procoagulant functions are conventionally known as "inhibitors".
- *Prevalence of inhibitors*: Up to 30% of patients with severe hemophilia A develop inhibitors to exogenously administered FVIII, while 3–5% of patients with severe hemophilia B develop inhibitors against exogenously administered FIX.
- *Predisposing factors for inhibitor development*: The risk of inhibitor development is a result of contribution from patient-related factors, genetic factors, and environmental factors (Table 12.4).
- *Impact of inhibitors on coagulation*: These antibodies are typically directed against functional epitopes on FVIII or FIX; hence, they neutralize the functional activity of exogenously administered clotting factor making the treatment ineffective.
- *Measurement of inhibitors*: The quantitative Bethesda assay is performed to assess the level of inhibitor (titer). One Bethesda unit (BU) is the amount of antibody that will neutralize 50% of FVIII or FIX in a 1:1 mixture of the patient's plasma and normal plasma (after 2 h incubation at 37 °C).
- *Clinical presentation of inhibitor*: Patients with inhibitors typically present with bleeding manifestations despite adequate prophylaxis, failure to achieve hemostasis with replacement therapy in the context of an acute bleed, or through routine screening. Low titer inhibitors (≤5 BU) are typically transient and may not have clinical bleeding manifestations. High titer inhibitors (>5 BU) are likely to persist and generally preclude effective treatment with factor replacement.

Table 12.3 Non-factor replacement products for patients with hemophilia

Product	Mechanism of action	Applicability to the types of hemophilia	Route of administration	Status
Emicizumab	Bispecific IgG antibody that possess FVIII mimetic properties	Only in hemophilia A	Subcutaneous	Approved for clinical use
Concizumab	Inhibits the action of TFPI, thereby releasing the "brakes" of the coagulation cascade	Both hemophilia A and B	Subcutaneous	Phase 2 trials
Fitusiran	Pairing with antithrombin III mRNA in hepatocyte results in inhibition of antithrombin synthesis	Both hemophilia A and B	Subcutaneous	Phase 3 trials

Table 12.4 Predisposing factors for the development of inhibitors in patients with hemophilia

Risk factor	Effect on inhibitor development
Patient related	Ethnicity: higher incidence in patients of African and Hispanic origin
	Family history of an inhibitor: higher incidence, if family history of inhibitors is present
	Age: highest incidence below 5 years. Increases after 60 years
Genetic risk factors	Severe hemophilia: large deletions, intron 22 inversion and nonsense mutations
	Mild and moderate hemophilia: certain mutations have a higher predisposition to development of inhibitors
	Different HLA class II alleles and polymorphisms in immune response genes either protect or predispose patients to inhibitor development
Treatment related	Previous exposure: highest risk is in the first 20–50 exposure days
	Intense first treatment: increases the risk
	Prophylaxis: early prophylaxis is associated with decreased risk
	Surgery: in severe hemophilia A with intense first treatment increases the risk of inhibitors. In mild or moderate cases with predisposing mutations, intensive treatment around surgery may increase the risk of inhibitors

- *Management of inhibitors*:
  - *Treatment of an acute bleeding episode*: It includes the use of "bypassing agents" such as activated prothrombin complex concentrates (APCC) or recombinant factor VIIa (rVIIa). APCCs contain multiple activated serine protease molecules such as activated forms of factor X and prothrombin to drive hemostasis without inhibition by FVIII or FIX inhibitors, whereas rVIIa is able to directly activate factor X and increase thrombin production on the surface of activated platelets in the absence of FVIII or FIX. Antifibrinolytics may also be used as adjunct therapy.
  - *Bleeding prophylaxis for inhibitors*: Clinical trials have demonstrated the efficacy of APCCs and rVIIa prophylaxis in reducing bleeding events for hemophilia with inhibitors. The substitution therapy, emicizumab, has demonstrated superior efficacy to bypassing agents within clinical trials, with a marked reduction in the treatment burden. However, breakthrough bleeding events still require treatment with bypassing agents. Caution is required when treating breakthrough bleeding events with bypassing agents, particularly with APCCs, as serious thrombotic complications were observed within the clinical trial program. A black box warning accompanies the use of emicizumab as part of risk mitigation for this complication.
  - *Eradication of inhibitors*: Immune tolerance induction regimens are used to eradicate the inhibitor. These regimens include frequent, often daily, infusions of high doses of factor concentrates with or without immunosuppressive agents. Patients with inhibitors should be treated at a specialized hemophilia treatment center (HTC). HTCs are federally recognized specialized clinics where a team of doctors, nurses, social workers, and physical therapists work together to deliver integrated care to persons with bleeding disorders. These centers have been shown to significantly decrease the morbidity and mortality for patients with bleeding disorders and have particular expertise to manage hemophilia with inhibitors. The list of these centers is available on the Centers for Disease Control and Prevention website (https://www.cdc.gov/ncbddd/hemophilia/HTC.html).

Gene Therapy for Hemophilia A and B

- This is fast emerging as an attractive option for severe hemophilia. The curative potential for gene therapy makes it a desirable option in both resource-limited and resource-rich settings [6].
- Currently the use of recombinant adeno-associated virus (AAV) as the vector to transfer the gene to the hepatocyte limits the use of this option to patients without pre-existing antibodies.
- Gene therapy has succeeded in converting patients with severe hemophilia to one with mild disease. However, through improved AAV technologies and use of hyperactive FIX mutants, recent gene therapy approaches have achieved factor VIII and IX activity levels maintained within the normal range (>50%) for at least 1 year.
- With the availability of extended half-life products, substitution, and hemostatic rebalancing therapies, the convenience of gene therapy will be compared with these products. Also, the long-term safety and efficacy of this potentially curative treatment will need to be established before it is used routinely in clinical practice.

von Willebrand Disease

Overview

von Willebrand Disease (vWD) is the most common inherited bleeding disorder resulting from a quantitative deficiency (type 1), qualitative dysfunction (types 2A, 2B, 2M, 2N), or absence (type 3) of vWF. In contrast to hemophilia, it is autosomally inherited, with types 1, 2A, 2B, and 2M inherited in a dominant manner and types 2N and 3 following a recessive pattern. Some reports suggest that vWD has a prevalence of about 1% in the general population, but the prevalence of clinically relevant cases is lower, ~100/million population [7].

Structure and Function of vWF

- *Synthesis*: vWF is a large multimeric glycoprotein that is synthesized in megakaryocytes and endothelial cells. It is stored within specific storage granules, the α-granules of platelets and Weibel-Palade bodies within endothelial cells. vWF is an acute phase reactant and the levels of vWF increase with physiological or pathological stress such as exercise, emotional stress, pregnancy, infections, and inflammatory conditions.
- *Structure*: The primary translation product contains a signal peptide of 22 amino acids, a propeptide of 741 residues, and the basic vWF monomer of 2050 amino acids. Each monomer contains 12 domains: 3 A domains (A1, A2, A3), 3 B domains (B1, B2, B3), 2 C domains (C1, C2), and 4 D domains (D1, D2, D'/D3) with a specific function; elements of note are:
  - The D′/D3 domain, which binds to FVIII
  - The A1 domain, which binds to platelet GPIb receptor and heparin
  - The A3 domain, which binds to collagen

  Two monomers of vWF are initially dimerized through disulfide bond formation at their C-termini. The C-terminal dimers are then N-terminal multimerized into multimers of 0.6 kD to 20 million Daltons. Unusually large vWF multimers that are synthesized and secreted by endothelial cells are processed into normal-sized multimers in the plasma through the action of a novel vWF-cleaving metalloprotease, ADAMTS13, which cleaves vWF within its A2 domain. This cleavage event is believed to occur under conditions of high shear stress in parts of the circulation, which partially unfolds the vWF molecule exposing the cleavage site. This physiologic regulation protects against abnormal platelet-vWF aggregation in the microvasculature, as seen in TTP and HUS (see Chap. 15.
- *Function*: High molecular weight (HMW) vWF multimers mediate platelet adhesion at sites of vascular injury by binding to subendothelial matrix and to platelets. vWF also serves as a carrier protein for plasma coagulation factor, FVIII. Therefore, defects in vWF can cause bleeding with features typical of platelet dysfunction, or of mild to moderately severe hemophilia A, or of both.

Genetics of vWD

- The *vWF* gene spans 178 kb in the human genome and is localized to the tip of the short arm of chromosome 12, at 12p13.3. In general, quantitative abnormalities of the *vWF* gene are due to promoter, frameshift nonsense mutations, and large deletions, whereas missense mutations typically result in qualitative defects. Mutations and polymorphisms in the *vWF* gene are currently being cataloged in an international database (https://grenada.lumc.nl/LOVD2/VWF/home.php?select_db=VWF).
- In several families, no mutations have been identified within the *vWF* gene implying the role of modifier genes in clinical expression of vWD. For example, ABO blood type is shown to be an important modifier of vWF plasma levels; patients with "O" type have lower plasma levels of vWF, while "AB" blood type is associated with the highest plasma levels of vWF.

Clinical Presentation

- In general, vWD is a mild bleeding disorder. Unlike hemophilia, spontaneous bleeding symptoms and bleeding within the internal organs such as CNS or joints are extremely rare (Table 12.5).
- The primary clinical presentation in patients with vWD is mucocutaneous bleeding such as:
  - Superficial bruising
  - Subcutaneous hematoma
  - Epistaxis
  - Gastrointestinal mucosal bleeding
  - Menorrhagia
  - Postpartum hemorrhage
  - Bleeding after common surgical procedures such as wisdom tooth extraction or tonsillectomy and adenoidectomy

Diagnosis of vWD

- Important components of the diagnosis of vWD include personal history of bleeding, family history of bleeding, and abnormal laboratory testing for vWD.
- Screening coagulation tests: PT and aPTT are usually normal; the aPTT may be prolonged due to low FVIII levels. Bleeding times and whole blood platelet function analyzers (PFA-100) are neither sensitive nor specific enough to rely on when screening for vWD; therefore, specific assays for vWF must be performed. The typical normal range of vWF:RCo and vWF:Ag is 50–150 IU per deciliter; however, cutoffs for clinical diagnosis may vary between centers.
- vWF laboratory assays:
  - *vWF antigen (vWF:Ag) assay*: This quantitative immunoassay measures the actual concentration of vWF protein in blood plasma.
  - vWF function analysis:

    The *ristocetin cofactor assay* (vWF:RCo) is a pharmacologic assay that measures the ability of vWF to bind platelet GPIb in the presence of ristocetin. The use of ristocetin in the diagnosis of vWD came

Table 12.5 Clinical characteristics of subtypes of von Willebrand disease and related disorder

Features	Type 1	Type 1C	Type 2A	Type 2B	Type 2N	Type 2M	Type 3	Platelet type vWD
Population frequency	1–2%	Rare	Rare	Rare	Rare	Rare	1:250,000	Rare
Defect	Partial quantitative deficiency with normal structure and function of vWF	Defect resulting in increased clearance of vWF	Quantitative and qualitative defect with loss of HMWM	↑ affinity of vWF to platelet membrane GP Ib/IX/V complex	↓ affinity of vWF for FVIII mimicking Hemophilia A	Qualitative defect with retention of HMWM	Complete deficiency of vWF	↑ affinity of platelet membrane receptor GP Ib/IX/V complex to vWF
Pattern of bleeding	Mucocutaneous	Mucocutaneous	Mucocutaneous	Mucocutaneous	Mucocutaneous, soft tissue, joint (rare)	Mucocutaneous	Mucocutaneous, soft tissue, joint	Mucocutaneous
Inheritance	AD	?AD	AD	AD	AR	AD	AR	AD
Platelet count	N	N	N	N or ↓	N	N	N	N or ↓
Factor VIII activity	N or ↓	N or ↓	N or ↓	N or ↓	↓ (discrepantly low compared to vWF antigen)	N or ↓	Markedly ↓	N or ↓
vWF Antigen (vWF:Ag)	↓	↓	↓	N or ↓	N	↓	Markedly ↓	N or ↓
Ristocetin cofactor activity (vWF R:Co)	↓	↓	↓	N or ↓	N	↓ (discrepantly low compared to vWF antigen)	Markedly ↓	N or ↓
Low-dose ristocetin-induced platelet aggregation (LDRIPA)	Absent	Absent	Absent	Present	Absent	Absent	Absent	Present
vWF multimer analysis	N	N	Absence of HMWM	Absence of HMWM	N	N or ↑ HMWM	Absent	Absence of HMWM
Treatment options	DDAVP Factor concentrate	Factor concentrate	DDAVP Factor concentrate	Factor concentrate	Factor concentrate	DDAVP Factor concentrate	Factor concentrate	Platelet transfusions

Adapted from Nelson Textbook of Pediatrics, 17th Edition, Chap. 469, Fig. 469-I
DDAVP desmopressin acetate, *HMWM* high molecular weight multimers, *AD* autosomal dominant, *AR* autosomal recessive, ↑ indicates prolonged, ↓ indicates decreased, *N* normal

about after patients being treated with this antibiotic were found to have developed a dose-dependent thrombocytopenia. Ristocetin, a positively charged antibiotic, induces structural change in vWF and exposes the domain for binding to platelet GPIb/IX/V complex. This assay is based on the ability of patient plasma to induce agglutination of formalin-fixed platelets in the presence of a fixed concentration of ristocetin. The level of vWF functional activity in a blood sample is assessed by comparing its ability to induce platelet agglutination with that of various dilutions of known normal pooled human plasma.

The adhesive function of vWF can also be measured by the *collagen-binding assay* (vWF:CBA), in which microtiter wells are coated with collagen which preferentially binds large vWF multimers.

- *Factor VIII activity*: Measured using a standard clotting factor assay based on the aPTT.
- *vWF multimer analysis*: These tests are available in specialized coagulation laboratories. The full range of vWF multimers within plasma are best demonstrated after fractionation on a low-concentration agarose gel. The vWF multimers can then visualized, for example, with radiolabeled anti-vWF antibody (Fig. 12.1).
- Therapeutic DDAVP challenge: This test is performed to document an appropriate response to DDAVP. For reasons not fully understood, there is a subset of patients whose vWF levels do not increase adequately following administration of DDAVP. Measuring sequential levels at baseline, followed by 1, 2, and 4 h post DDAVP administration, can identify patients who show an adequate initial response but subsequent rapid clearance (type 1C).
- Specialized assays:

  Following a diagnosis of vWD, additional functional assays may be used to determine the affinity of vWF for FVIII or platelets. These assays may assist in determining certain qualitative subtypes of vWD.

  Ristocetin-induced platelet aggregation (RIPA): This assay is similar in principle to the test performed for vWF:RCo activity, except that the patient's own platelets are used to evaluate the sensitivity of the patient's vWF platelet aggregation in response to both low- and high-dose ristocetin.

  von Willebrand factor propeptide (vWFpp): vWF propeptide and vWF are synthesized in a 1:1 ratio. An increase in the ratio toward the propeptide suggests an increased clearance of vWF (type 1C).

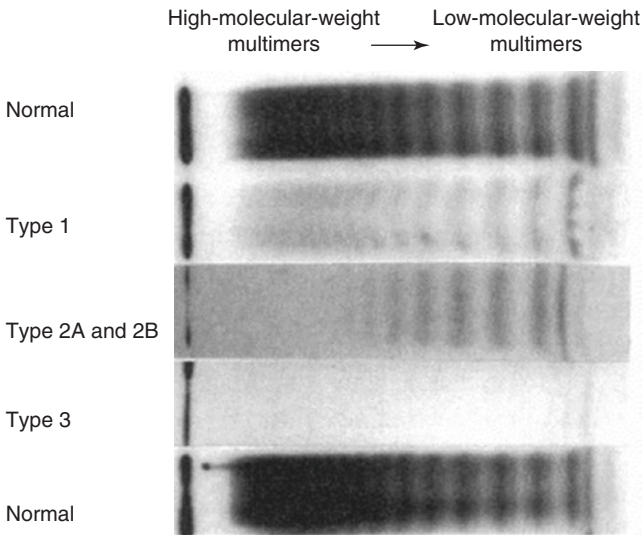

Fig. 12.1 von Willebrand multimer analysis. (Reprinted from Pruthi [14])

- Interpretation of vWF diagnostic assays: vWF antigen and functional assays should be interpreted in the context of vWF multimer assays. In general, a proportionate decrease in vWF antigen, vWF functional activity, and FVIII activity accompanied by a normal distribution of multimers of vWF (from low to high molecular weight) indicates a quantitative defect. Lack of concordance of the functional and antigen assays (<0.5–0.6) and/or an abnormal distribution of vWF multimers indicates a qualitative defect. Additional specialized assays may be used to identify the specific qualitative defect. Since there is no single specific test for vWD, an array of testing is necessary. Functional assays looking for collagen binding and GPIb affinity are useful. Genetic testing is very useful and should be used to recognize and clarify type 2 patients. Importantly, not only are there variants of vWF, but there are other contributors to laboratory variability. For example, vWF levels may be elevated due to anxiety in a pediatric patient at the time of blood draw or related to estrogen levels in females. It is most useful to collect vWF samples for diagnostic testing on the 1st day after a menstruating woman has completed her menses.

Clinical Subtypes of vWD

- *vWD subtypes:* vWD is classified into three major subtypes: type 1, type 2, and type 3 (Table 12.5). It is best understood as either quantitative deficiency (type 1 or type 3) or a qualitative deficiency (types 2A, 2B, 2N, 2M). Patients who rapidly clear vWF are classified as type 1C.
- *Prevalence of vWD subtypes*: Most cases (~85%) appear to have a partial quantitative deficiency of vWF (type 1 vWD) with variable bleeding tendency. About 20–30% patients have qualitative variants (type 2 vWD), due to a

dysfunctional vWF. These patients are clinically more homogeneous and exhibit moderate bleeding tendency. Type 3 vWD is rare (~5%), and the patients have a moderate to severe bleeding diathesis because of the virtual absence of vWF and concurrent deficiency of FVIII.

- *Mode of inheritance:* Types 1, 2A, 2B, and 2M are inherited in an autosomal dominant fashion, while type 2N and type 3 are inherited in an autosomal recessive fashion.
- *Pathophysiology and its implications on management*:
  - Type 1 vWD: This subtype may be caused by mutations that result in decreased synthesis or promote increased clearance of vWF from circulation. Proportionate reductions in vWF:Ag, vWF:RCo, and FVIII activity and a normal distribution of vWF multimers are consistent with type I vWD. These patients respond to pharmacological agents such as DDAVP which acts to raise endogenous levels of vWF through release from endothelial storage granules.
  - Type 1C vWD: This subtype, which has been recently described, consists of an increased clearance of vWF. While these patients will initially respond to DDAVP with an appropriate increase in vWF levels, the vWF is subsequently rapidly cleared from the circulation. Knowing a patient's response to DDAVP helps tailor the treatment options.
  - Type 2A vWD: This is typically caused by mutations that interfere with the assembly or secretion of high molecular weight (HMW) multimers of vWF or that increase the susceptibility of vWF to proteolysis. This results in a disproportionate reduction in vWF:RCo compared to vWF:Ag and deficit of HMW multimers. Although these patients may respond to DDAVP, vWF concentrates are often required for the treatment of moderate to severe bleeding.
  - Type 2B vWD: This subtype is caused by a "gain of function" mutation in the A1 region of vWF leading to spontaneous binding of vWF to platelets which leads to proteolytic degradation and depletion of the HMW multimers and platelets. These patients typically present with thrombocytopenia, reduced vWF levels, and absence of HMW multimers. The laboratory diagnosis relies on the demonstration that the "hyperreactive" vWF binds to platelets resulting in agglutination of platelets in the RIPA at low concentration of ristocetin. Since DDAVP can release these "hyperreactive" vWF multimers, this can worsen the thrombocytopenia and should be used with caution in this subtype of vWD. These patients are usually treated with vWF concentrates. Type 2B vWD must be distinguished from platelet-type vWD or pseudo-vWD where mutations within the GP1b/IX/V platelet receptor results in increased binding of platelets to vWF. Since the defect is in the platelets, standard treatment options for vWD are not effective for platelet-type vWD. Such patients may require treatment with normal platelets to control bleeding. Genetic testing is useful for type 2B patients.
  - Type 2M: This describes vWF variants that exhibit decreased vWF-dependent platelet adhesion despite the presence of HMW multimers. This results in the vWF:RCo assay being disproportionately decreased when compared to vWF:Ag. The distinction from type 2A vWD depends on vWF multimer analysis. Pharmacological treatment with DDAVP is generally effective for the treatment of bleeding manifestations.
  - Type 2N: This type is caused by genetic mutations in the FVIII binding domain of vWF. This results in rapid clearance of FVIII from plasma and subsequently low residual FVIII plasma levels. This subtype of vWD has been referred to as "autosomal hemophilia" or the "Normandy" variant. Patients will have normal vWF:Ag and vWF:RCo levels but decreased FVIII activity (<10%). The laboratory diagnosis relies on a specific binding assay that confirms the decreased binding affinity of vWF for FVIII. Multimer analysis will be normal. The majority of patients will still respond to DDAVP treatment as FVIII is released from storage granules along with vWF, though the half-life of the endogenous FVIII will remain very short. If FVIII levels are <5%, then treatment with vWF-containing FVIII concentrates should be considered to treat major bleeding events. Genetic testing is confirmatory in these patients.
  - Type 3 vWD: This subtype is characterized by undetectable levels of vWF:Ag and vWF:RCo and complete absence of vWF on multimer analysis. FVIII levels are usually very low (1–9%). These patients have the most severe bleeding manifestations and may exhibit a clinical phenotype similar to moderate hemophilia A. These patients do not respond to DDAVP and require vWF concentrates for the treatment of bleeding.

Treatment of vWD

- The principles of treatment of bleeding in patients with vWD include increasing functional vWF levels and enhancing clot stability.
- Specific treatment options to raise vWF levels include DDAVP and vWF concentrates. Adjuvant therapies such as antifibrinolytics and local hemostatic agents are used to enhance clot stability. Hormone suppression therapy can be used to control menorrhagia; estrogens are also shown to increase vWF levels. Table 12.5 illustrates the details of pharmacotherapy.
- *DDAVP*: DDAVP is generally used as a first line of therapy in cases of mild bleeding or bleeding in the oral cavity

in patients with type 1, 2A, 2M, and 2N VWD with or without adjuvant therapies. It is available as an intravenous preparation and as an intranasal spray.

- *Plasma-derived VWF concentrates*: These contain HMW multimers of vWF as well as FVIII. These are usually recommended for the treatment of severe bleeding in patients with vWD or for any other bleeding episodes which are unresponsive to other therapies. Similar to hemophilia, for major bleeding episodes, the levels of VWF should be raised to 80–100%, and for minor bleeding events, the level should be raised up to 30–50%. The dosing of vWF concentrates is calculated based on the ristocetin cofactor units. One unit per kg of ristocetin cofactor activity raises the plasma vWF:RCo activity by ~1%. The half-life of vWF:RCo activity after infusion is ~10 to 12 h. Further maintenance treatment with vWF concentrates depends upon the severity of bleeding and the initial response to factor concentrate treatment.
- *Human recombinant vWF*: This is a recombinant analog of vWF with only trace amount of FVIII. It is manufactured in the absence of animal or human-derived proteins. It contains ultra large molecular weight multimers in addition to the high, medium, and low molecular weight multimers that are normally found in plasma. This agent can be used for treating both acute bleeding episodes and in the perioperative management of bleeding. For minor bleeds, a dose of 40–50 IU/kg is recommended followed by repeating it every 8–24 h. For major bleeds, a dose of 50–80 IU/kg is recommended followed by 40–60 IU/kg every 8–24 h. Some acute bleeds might require a co-infusion of rVWF and rFVIII in the ratio of 1.3:1. This achieves immediate hemostatic levels of FVIII [9].

Acquired Abnormalities of vWF

Alterations in the amount and function of vWF have been reported in following pathologic states:

- Acquired von Willebrand syndrome secondary to autoantibody formation is described in autoimmune disorders (SLE or scleroderma), lymphoma, leukemia, multiple myeloma, and monoclonal gammopathies. These antibodies may inhibit the function of vWF and/or reduce the half-life of vWF by enhancing its clearance [10].
- Wilm's tumor due to adsorption of vWF on tumor cells.
- Hypothyroidism due to decreased synthesis of vWF.
- Congenital heart disease such as ventriculoseptal defect, aortic stenosis, interrupted aortic arch, and pulmonary hypertension may cause loss of the HMW multimers of vWF and may mimic type 2A.
- Left ventricular assist device increases shear stress and oxidative stress that enhances the binding of vWF to platelets. This increases the clearance of HMW multimers.
- Vascular malformations (angiodysplasia, giant hemangiomas, and telangiectasia) have been associated with abnormal synthesis and clearance of vWF multimers.

Rare Bleeding Disorders

Overview

- Qualitative and quantitative deficiencies of coagulation factors such as fibrinogen, prothrombin, factor V, combined deficiency of factors V and VIII, factor VII, factor X, factor XI, factor XIII, and multiple deficiencies of vitamin K-dependent coagulation factors are collectively known as rare bleeding disorders (RBD).

Epidemiology

- These disorders are usually transmitted in an autosomal recessive manner. Therefore, they can affect both males and females equally.
- The prevalence of RBD is low ranging from 1:500,000 for FVII deficiency to 1:2,000,000 for prothrombin and factor XIII deficiency.
- Ethnic/racial predisposition: The prevalence of RBDs is significantly increased by a high rate of consanguinity in the population, such as Middle Eastern communities. FXI deficiency is more commonly seen in Ashkenazi Jews.
- Rare bleeding disorders appear to be underreported in parts of the world that lack appropriate healthcare infrastructure with a majority of cases reported from North America and Europe.

Bleeding Manifestations

- In general, the bleeding patterns among patients with RBDs are quite variable, and, unlike hemophilia, the coagulation factor levels do not necessarily correlate with the severity of the bleeding [11].
- There is a strong correlation between residual factor level and symptoms in patients with fibrinogen, combined FV + FVIII, FX, and FXIII deficiency. A weaker correlation is observed for patients with FV and FVII deficiencies and no association in those with FXI deficiency.
- In heterozygotes, with coagulant activity of >30%, postoperative bleeding and mucocutaneous bleeding have been noted in 20% of patients.
- Overview of bleeding symptoms: The majority of these patients can be asymptomatic and present with hemorrhages after hemostatic challenge such as menstruation and surgical procedures. Rarely patients with severe deficiency can present with symptoms of mucocutaneous

bleeding, hemarthrosis, and deep muscle hematoma. Life-threatening bleeding events, such as CNS bleeds, appear to be less frequent than in patients with hemophilia, except in patients with fibrinogen, factor VII, and factor XIII deficiency. About 24% of patients with severe factor XIII deficiency (FXIII <1%) present with spontaneous CNS bleeds in the newborn period. Besides bleeding symptoms, patients with dysfibrinogenemia and afibrinogenemia can present with strokes or thromboembolic events. Women with dysfibrinogenemia or afibrinogenemia and factor XIII deficiency can present with miscarriages.

Diagnosis of RBDs

- Detailed clinical history and a high index of suspicion are the keys to diagnose RBDs. Many of these patients are diagnosed either due to the positive family history or history of unexpected bleeding when exposed to hemostatic challenge such as menstruation, childbirth, and surgical procedures [12].
- Mild factor deficiencies are usually diagnosed by measuring the factor level and may not affect the PT and aPTT testing. Factor XIII deficiency does not affect the PT or aPTT.
- Thrombin time (TT) is prolonged with fibrinogen deficiency, and many dysfibrinogenemias associated with bleeding such as fibrinopeptide A release defects and polymerization defects.
- In patients with suspected functional abnormalities of the proteins such as dysfibrinogenemia and dysprothrombinemia, immunological assays quantitate the antigen level in addition to measurement of functional levels. Since the amount activity and antigen of a protein should be the same, the presence of reduced activity in relation to the total amount of antigen indicates an abnormal molecule.
- Factor XIII deficiency: Since all the screening tests are normal in patients with factor XIII deficiency, a specific assay, the 5 M urea clot solubility assay, is required to recognize factor XIII deficiency. Since factor XIII is required for the cross-linking of fibrin monomers, this diagnostic test is based upon the observation that there is an increased solubility of the clot because of the failure to cross-link the fibrin monomers. The normal clot remains insoluble in the presence of 5 M urea, whereas the clot formed from a patient with factor XIII deficiency dissolves rapidly within hours. It is important to note that this method detects only severe factor XIII deficiency (with activity typically below 1%). Functional and immunologic assays for factor XIII quantitation are also available in specialized laboratories and measure milder forms of factor XIII deficiency.
- Scope of genetic testing for the diagnosis of RBDs: Unlike hemophilia, the molecular pathology of RBDs is not well described. Therefore, routine genetic testing for the diagnosis of RBDs is not available for clinical use.

Management of RBDs

- Similar to hemophilia, the management of RBDs focuses on treatment of acute bleeding events through factor replacement therapy. When factor concentrates are not available, fresh frozen plasma and cryoprecipitate are appropriate treatment options. In general 30% levels of factor XI and factor V are sufficient in deficient patients to achieve surgical hemostasis [13].
- Unlike hemophilia, most rare bleeding disorders do not require prophylactic factor replacement. Certain patients with afibrinogenemia, FXIII and severe FVII deficiency may benefit from prophylaxis.
- Therapeutic options for patients with RBD should depend on the nature of the bleed. Minor and mucocutaneous bleeds can be managed with antifibrinolytics. Tranexamic acid is relatively contraindicated in renal tract bleeding since it is associated with renal vein thrombosis and ureteral clots. Antifibrinolytics always are contraindicated in patients with disseminated intravascular coagulation (DIC) since its use can be associated with myocardial infraction, stroke, and renal vein thrombosis.
- Multiple dosing with prothrombin concentrate complex (PCC) also may be associated with a prothrombotic risk.
- Specific concentrates for fibrinogen, factor VII, factor X, and factor XIII are available in the USA (Table 12.6). Factor XI concentrate is available in Western Europe. PCC contains factors II, VII, IX, and X and may be suitable for deficiencies of prothrombin or factor X. For other RBDs, fresh frozen plasma remains an option for the treatment or prevention of major bleeding events. Conventionally, 10–20 mL/kg of FFP will often increase the factor levels by 10–20%. Volume overload and the potential for transmission of blood-borne pathogens discourage their routine use. FXI concentrate replacement over 30% has been associated with thrombosis in FXI deficient patients.

Miscellaneous Bleeding Disorders: Deficiencies of Inhibitors of Fibrinolytic Pathway

- Deficiency of either of the two inhibitors of plasmin, α-2 antiplasmin and plasminogen activator inhibitor-1 (PAI-1), results in increased plasmin generation and premature lysis of fibrin clots leading to a bleeding tendency.
- Clinical symptoms: These patients experience mucocutaneous bleeding but rarely have joint hemorrhages.
- Diagnosis: The commonly used screening coagulation tests are normal in patients with these RBDs. Specific

Table 12.6 Characteristics and management of rare bleeding disorders

Factor deficiency	Mode of inheritance	Site of production	Plasma half-life	Diagnostic test	Do factor levels correlate with symptoms?	Clinical features specific to the condition	Treatment products available
Hypoprothrombinemia	AR	Liver	3–4 days	Prolonged PT and APTT	Insufficient data	Absent prothrombin is incompatible with life	Prothrombin complex concentrate
Dysprothrombinemia				F II activity			
				F II antigen		Rarest RBD	
F V deficiency	AR	Liver and possibly platelets	36 h	Prolonged PT and PTT	Good correlation		FFP
				F V activity			Platelet transfusion (20% FV is stored in platelets)
							Plasma-derived FV in preclinical studies
F V + FVIII deficiency	AR	FV: liver	FV: 36 h	Prolonged PT and APTT (APTT disproportionately prolonged)	Excellent correlation	Mild to moderate bleeding tendency	DDAVP
		FVIII: endothelium	FVIII: 10–14 h	F V and F VIII activity			FVIII concentrate
F VII deficiency	AR	Liver	4–6 h	Prolonged PT with normal APTT	Good correlation	Most common RBD	Plasma-derived factor concentrate
				F VII assay			Recombinant factor concentrate
F X deficiency	AR	Liver	40–60 h	PT and APPT is prolonged	Excellent correlation	Most severe RBD	PCC
				F X assay		CNS bleed	Plasma-derived factor concentrate
						Umbilical cord bleed	
						GI bleed	
F XI deficiency	AR/AD	Liver	50 h	Normal PT and prolonged APTT	No correlation	Second most common RBD	FFP
				Mixing study: correction		High prevalence among Ashkenazi Jews (1:450)	Plasma-derived factor concentrate (only in Europe)
				F XI assay			
Afibrinogenemia	AR/AD	Liver	2–4 days	Prolongation of PT, APTT and TT	Excellent correlation	Spontaneous abortions	Cryoprecipitate
Hypofibrinogenemia				Mixing study: correction		Thrombotic risk as fibrin activates the fibrinolytic pathway	Plasma-derived factor concentrate
Dysfibrinogenemia				Fibrinogen antigen			
				Fibrinogen activity			
				DRVVT			

FXIII deficiency	AR	A subunit: megakaryocytes and monocytes	9–12 days	Normal PT and APTT	Excellent correlation	Spontaneous abortions	Cryoprecipitate
		B subunit: liver		Urea clot solubility test: early clot lysis		Increased incidence of intracranial hemorrhage in neonates	Plasma-derived factor concentrate
				F XIII quantitative analysis		Delayed wound healing	Recombinant factor concentrates (rXIII-A)
PAI 1 deficiency	AR	Endothelial cells		Normal PT and PTT	Unclear correlation	Recurrent miscarriages	Antifibrinolytics
		Megakaryocytes		Shortened euglobulin clot lysis time (ECLT)		Delayed bleeding	
		Liver					
		Adipocytes		Antigenic and chromogenic PAI 1 assay			
α2-antiplasmin deficiency	AR	Liver		Normal PT and APTT	Unclear correlation	Recurrent miscarriages	Antifibrinolytics
		Kidney		Decreased α2-antiplasmin activity with decreased or normal antigen		Delayed bleeding	FFP
						Intramedullary hemorrhage	

tests such as the euglobulin clot lysis time that measures fibrinolytic activity are shortened in the presence of these deficiencies. Specific assays for α-2-antiplasmin and PAI-1 quantitation available at specialized laboratories need to be measured.

- Treatment of bleeding: Antifibrinolytic drugs such as epsilon aminocaproic acid and tranexamic acid are the cornerstones of management of bleeding in these patients (Table 12.6). Rarely FFP is used as a source of these proteins to control severe bleeding.

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Acquired Bleeding Disorders

13

Marcel Levi

Introduction

Defects in the hemostatic system can result in a bleeding disorder. These defects can be either congenital or acquired. Acquired bleeding disorders are common in clinical practice, and it is important to differentiate between the various underlying mechanisms as they may require different therapeutic interventions. This chapter focuses on the acquired bleeding disorders and will review how careful history, physical examination, and laboratory evaluation help in the differential diagnosis and management of these defects. A list of the various acquired coagulation defects is given in Table 13.1.

Diagnosis

Medical History and Bleeding History

The cornerstone for recognition of a clinically significant coagulation disorder is the medical history, which should include an inquiry specifically about previous surgical procedures, bleeding complicating trauma, and bleeding after tooth extraction [1]. While most congenital coagulation disorders might be identified on the basis of a history of lifelong bleeding complications after minor trauma or interventions and a bleeding tendency in other members of the family, patients with mild hemostatic defects may not experience major hemorrhage until adulthood after challenge. In addition, the history should particularly focus on the use of drugs that might affect the coagulation system. The medical history should focus on the history of other medical conditions that may lead to coagulation defects, such as hepatic dysfunction and renal failure. Patients with autoimmune disorders, such as rheumatoid arthritis or systemic lupus erythematosus, or with lymphoproliferative diseases, may develop antibodies against platelets or coagulation factors.

Physical Examination

During physical examination, abnormal bruising, petechiae, and splenomegaly are signs that might point to a defect in the coagulation system. Mucosal bleeding and petechiae are most likely associated with a defect in primary hemostasis, whereas muscle and joint bleeding typically occur in patients with a severe defect in fibrin formation. The term "primary hemostasis" refers to events mediated by platelet or von Willebrand factor function (see Chap. 10 for a discussion about this issue). Jaundice, ascites, spider nevi, and splenomegaly point into the direction of liver cirrhosis with impaired synthesis of coagulation factors and thrombocytopenia due to sequestration in the enlarged spleen. Signs and symptoms of sepsis or cancer may be associated with disseminated intravascular coagulation.

Laboratory Tests

Retrospective and prospective studies have shown that in case of a negative bleeding history and a normal physical examination, routine coagulation tests, for example, preceding surgical procedures, are not useful, with the possible exception of very extensive surgery or surgery that poses a particularly great challenge to hemostatic competence (e.g., neurosurgical procedures) [2]. Screening coagulation tests include a platelet count and measurement of activated partial thromboplastin time (aPTT) and prothrombin time (PT). The bleeding time is not used anymore because the test is highly operator- and situational- (room temperature, skin circulation, etc.) dependent and is not sufficiently reliable to be useful in the diagnostic process.

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Table 13.1 Overview of most important acquired bleeding disorders

Primary hemostasis	Fibrin formation	Primary hemostasis and fibrin formation
Thrombocytopenia	Global low levels of coagulation factors	Advanced liver disease
Bone marrow disease	Impaired synthesis due to liver disease	
Drug-induced thrombocytopenia	Immune thrombocytopenia	
Infection/sepsis	Impaired synthesis due to vitamin K deficiency	
Thrombotic microangiopathy	Massive loss	
Heparin-induced thrombocytopenia	Dilution after plasma expander treatment	
Qualitative platelet defect	Inhibiting antibodies toward a coagulation factor (e.g., acquired hemophilia)	Consumption coagulopathy (disseminated intravascular coagulation)
Uremia		
Liver disease		
HIV		
Immunoglobulinopathies		
Acquired von Willebrand disease	Anticoagulants	
	Heparin and heparin derivatives	
	Vitamin K antagonists	
	Direct oral anticoagulants	
	Anti-Xa agents	
	Anti-IIa (thrombin) agents	
Drugs		
Aspirin		
Thienopyridine derivatives		
Glycoprotein IIb/IIIa inhibitors		

It is important to emphasize that the global coagulation tests poorly reflect in vivo hemostasis. However, these tests are a convenient method to quickly estimate the concentration of one or at times multiple coagulation factors for which each test is sensitive [3]. In general, coagulation test results will be prolonged if the levels of coagulation factors are below 50%. The normal values and the sensitivity of these tests for deficiencies of coagulation factors may vary markedly between tests, depending upon the reagents and equipment used. Therefore, an increasing number of laboratories use the international normalized ratio (INR) instead of the prothrombin time. While this may allow for greater standardization between centers, it should be mentioned that the INR has only been validated for control of the intensity of vitamin K antagonist therapy [4].

Table 13.2 summarizes the interpretation of a prolonged aPTT, PT, or both in patient with an acquired bleeding disorder [5]. A prolonged aPTT as a sole abnormality can be caused by a deficiency of factor VIII, IX, XI, or XII, prekallikrein, and high molecular weight kininogen or presence of heparin or by an inhibitor, which can be either factor specific, such as an antibody against factor VIII, or non-specific, such as the presence of a lupus anticoagulant. A prolonged PT as the sole finding can indicate a factor VII deficiency, or a mild vitamin K deficiency with defects in factors X or II, or a mild dysfibrinogenemia. Abnormalities of both PT and aPTT may indicate a deficiency of fibrinogen, prothrombin, factor V or factor X, an inhibitor to one of these factors, a severe vitamin K deficiency or use of vitamin K antagonists, or a combined deficiency of coagulation factors due to impaired synthesis, massive loss of coagulation factors, or a consumptive coagulopathy. Dependent of the result of these tests, specific assays for individual coagulation factors may be performed. Vitamin K deficiency may be diagnosed by measuring a vitamin K-dependent coagulation factor (e.g., factor VII) and a non-vitamin K-dependent factor (e.g., factor V) and by observing normalization of the coagulation times after administration of factor VII.

Table 13.2 Interpretation of global coagulation tests in acquired coagulation defects

Test result	Cause
PT prolonged, aPTT normal	Mild vitamin K deficiency
	Mild liver insufficiency
	Low doses of vitamin K antagonists
	Low concentration of oral factor Xa inhibitors
PT normal, aPTT prolonged	Acquired factor VIII deficiency
	Other inhibiting antibody and/or antiphospholipid antibody
	Use of unfractionated heparin
Both PT and aPTT prolonged	Acquired factor V deficiency
	Severe vitamin K deficiency
	Higher doses of vitamin K antagonists
	Global clotting factor deficiency
	Synthesis: liver failure
	Loss: massive bleeding
	Consumption: DIC

To distinguish between a deficiency state and the presence of an inhibitor, repeating the abnormal test, the PT, and/or the aPTT, a 1:1 mixture of the patient's plasma and normal plasma

is useful. If the mixture normalizes the prolonged PT or aPTT, a deficiency state is likely, as most coagulation tests are calibrated to produce a normal result if each of the relevant factor levels are 50% of normal or greater. If the mixture still yields a significantly prolonged PT or aPTT or does not correct into the normal range for the assay, an inhibitor probably is present.

Detection of heparin can be done utilizing the aPTT; however, this test is not sensitive for low molecular weight (LMW) heparin. In that case the anti-factor Xa chromogenic assay is more useful. This assay can also be employed for detection of the direct oral anticoagulants, such as rivaroxaban or apixaban. None of the currently available laboratory tests is useful in monitoring dabigatran, which can only be done by performing an ecarin clotting time or an anti-factor IIa chromogenic assay; however, that test is usually not readily available in a routine setting.

Acquired Platelet Disorders: Impairment of Primary Hemostasis

Quantitative Defects in Primary Hemostasis: Thrombocytopenia

Table 13.3 summarizes the most frequently occurring diagnoses in patients with an acquired thrombocytopenia (see Chap. 15). Thrombocytopenia is relatively frequently caused by a bone marrow problem (hematologic malignancy, repression of megakaryocytes due to bone marrow infiltration, myelosuppressive effects of cytostatic, or otherwise myelotoxic agents). In all these cases, severe thrombocytopenia is treated by platelet transfusion.

In addition, immune thrombocytopenia (as an isolated disorder or in the framework of a systemic condition such as rheumatoid arthritis, lupus erythematosus, or HIV) is a common clinical condition. Important components in the management of immune thrombocytopenia are corticosteroids, immunoglobulins, thrombopoietin-like growth factors, and in some cases more advanced immunomodulatory agents, such as anti-CD20 antibodies.

Other clinical conditions associated with thrombocytopenia are severe infections and sepsis. Thrombocytopenia is a strong marker for the severity of sepsis [6]. The principal factors that contribute to thrombocytopenia in patients with sepsis are impaired platelet production, increased consumption or destruction, or sequestration of platelets in the spleen. In a substantial number of patients with sepsis, marked hemophagocytosis may occur (Fig. 13.1) [7]. Platelet consumption probably also plays an important role in patients with sepsis. Thrombin is the most potent activator of platelets in vivo, and intravascular thrombin generation is a ubiquitous event in sepsis with or without evidence of overt disseminated intravascular coagulation (see further).

Table 13.3 Differential diagnosis of acquired thrombocytopenia

Differential diagnosis	Additional diagnostic clues
Bone marrow failure	Low hemoglobin/low red cell count, low white blood cell count, low number of megakaryocytes in bone marrow aspirate
Immune thrombocytopenia	Decreased number of megakaryocytes in bone marrow aspirate or detection of drug-induced antiplatelet antibodies, rebound of platelet count after cessation of drug
Sepsis from any etiology	Platelet consumption occurs commonly from bacterial, viral, fungal, and rickettsial sepsis
Disseminated intravascular coagulation (DIC)	DIC produces small fibrin fragments that have the ability to bind platelet integrins and function as adhesive glycoproteins inducing platelet aggregation
Drug-induced thrombocytopenia	Antiplatelet antibodies, normal or increased number of megakaryocytes in bone marrow aspirate, thrombopoietin (TPO) decreased
Massive blood loss	Major hemorrhage, low hemoglobin, prolonged aPTT and PT
Thrombotic microangiopathy	Schistocytes in blood smear, coombs-negative hemolysis, fever, neurologic symptoms, renal insufficiency
Heparin-induced thrombocytopenia	Use of heparin, venous or arterial thrombosis, positive HIT test (usually ELISA for heparin-platelet factor IV antibodies), rebound of platelets after cessation of heparin

The group of thrombotic microangiopathies encompasses syndromes such as thrombotic thrombocytopenic purpura (TTP), hemolytic-uremic syndrome (HUS), severe malignant hypertension, and chemotherapy-induced microangiopathy [8] and is more extensively discussed in Chap. 15 (acquired thrombocytopenias). Briefly, a common pathogenetic feature of these clinical entities appears to be endothelial damage, which causes platelet adhesion and aggregation. The multiple clinical consequences of this extensive endothelial dysfunction include thrombocytopenia, mechanical fragmentation of red cells with hemolytic anemia, and obstruction of the microvasculature of the kidney, brain, and other organs (leading to renal failure and neurological dysfunction, respectively). Despite this common final pathway, the various thrombotic microangiopathies have different underlying etiologies. Thrombotic thrombocytopenic purpura is caused by deficiency of von Willebrand factor cleaving protease (ADAMTS-13), resulting in endothelial cell-attached ultra-large von Willebrand multimers that readily bind to platelet surface receptors and cause platelet adhesion and aggregation [9]. In hemolytic-uremic syndrome, a cytotoxin released upon infection with a specific serogroup of Gram-negative microorganisms (usually *E. coli* serotype O157:H7) is responsible for endothelial cell and platelet activation. In cases of malignant hypertension or chemotherapy-induced thrombotic

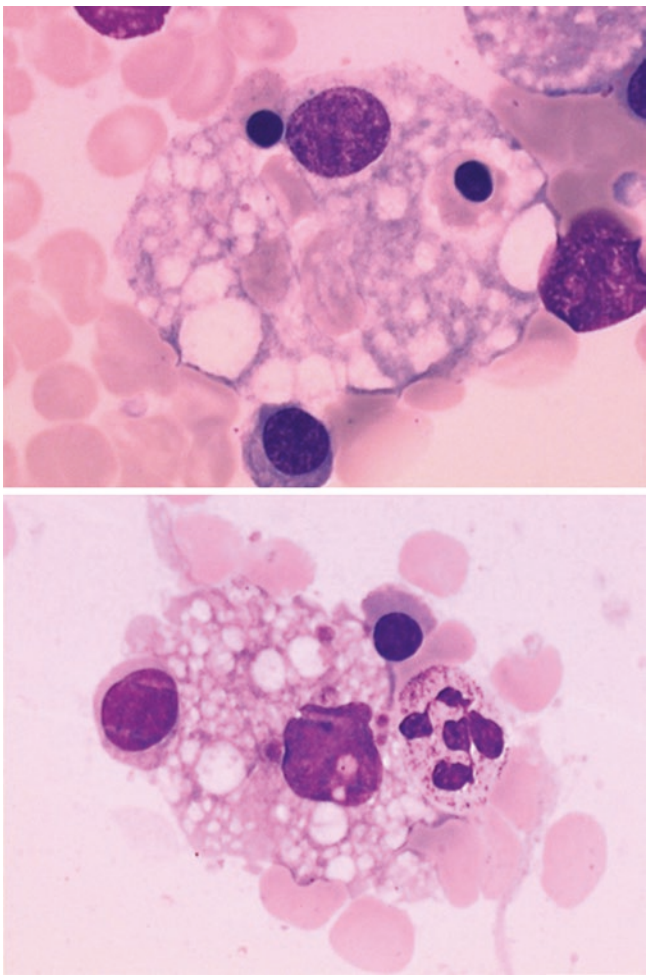

Fig. 13.1 Typical examples of hematophagocytosis of bone marrow cells by macrophages. The bone marrow was obtained from a patient with severe sepsis (May-Grunwald-Giemsa staining, ×500) (Courtesy of Bruno Francois and Frank Trimoreau, Dupuytren Hospital, Limoges, France)

microangiopathy, direct mechanical or chemical damage to the endothelium may be responsible for the enhanced endothelial cell-platelet interaction [10]. Currently, the cornerstone in the treatment of TTP and HUS is plasmapheresis.

Heparin-induced thrombocytopenia (HIT) is caused by a heparin-induced antibody that binds to the heparin-platelet factor 4 complex on the platelet surface [11, 12] (see Chap. 15 (acquired thrombocytopenias)). This may result in massive platelet activation followed by a consumptive thrombocytopenia and arterial and venous thrombosis. The incidence of HIT may be as high as 5% of patients receiving heparin and is dependent upon the type and dose of heparin and the duration of its administration (usually more than 7 days but may be sooner in patients treated with heparin in the previous 3 months). Immediate cessation of heparin and initiation of alternative non-heparin and non-vitamin K antagonist anticoagulation (e.g., with direct thrombin inhibitors or other direct oral anticoagulants) is crucial in patients with HIT.

Qualitative Defects in Primary Hemostasis

Acquired qualitative platelet defects may occur in patients with uremia. Although the underlying pathogenesis is not exactly known, anecdotal evidence seems to indicate the platelet dysfunction is not caused by urea per se [13]. Part of the increased bleeding tendency in patients with chronic renal failure may be caused by the relatively low hematocrit that can cause an impaired platelet adhesion due to changed rheological conditions in the blood; however, in patients adequately treated with erythropoietin, administration this factor has become less relevant. In addition, administration of desmopressin is effective in improving primary hemostasis in patients with uremic thrombocytopathy.

Other systemic conditions that cause impaired primary hemostasis are severe liver dysfunction and high levels of immunoglobulins, which may occur in myeloma or Waldenström macroglobulinemia and which can derange platelet function due to non-specific binding to fibrinogen, platelets, and blockage of platelet receptors.

Acquired von Willebrand factor (vWF) deficiency is a relatively rare condition and usually presents in combination with other conditions, such as essential thrombocythemia, hypothyroidism, lymphoproliferative disorders, or other malignancies [14]. Several drugs, including ciprofloxacin and valproic acid, have been associated with acquired VWD. The disease is caused by autoantibodies to VWF triggering rapid clearance of VWF from the circulation and sometimes by interfering with VWF function. Bleeding in patients with acquired VWD is managed by (high dose) vWF concentrate, desmopressin, or by recombinant factor VIIa. Treatment is usually directed to the underlying disorder. Refractory patients have been managed with corticosteroids, plasma exchange, or intravenous immunoglobulin (IVIG).

Drugs that inhibit platelet function are important agents in the management of arterial thromboembolic disease and very frequently used for secondary prevention in patients with coronary artery disease or stroke. Low-dose aspirin is one of the most frequently used drugs worldwide. Aspirin, an irreversible platelet inhibitor by acetylation of cyclooxygenases' two active sites, increases the risk of bleeding, in particular gastrointestinal bleeding, and has been associated with a small but consistent increase in intracerebral hemorrhage. Over the last few years, the approach to the patient who uses aspirin and who presents with bleeding or needs to undergo an invasive procedure has changed considerably. In fact, in current clinical practice, bleeding can almost always be managed with local hemostatic procedures or conservative strategies without interrupting aspirin, and also most invasive procedures do not require the cessation of aspirin when adequate attention is given to local hemostasis [15]. In contrast, interruption of aspirin has been associated with an increased risk of thromboembolic complications, potentially

due to a rebound hypercoagulability. Obviously, in special clinical circumstances, such as intracranial bleeding or the need to undergo a neurosurgical or ophthalmic procedure, the anti-hemostatic effect of aspirin needs to be reversed immediately. The most rigorous measure to achieve that is the administration of platelet concentrate after cessation of aspirin or administration of desmopressin.

Other cyclooxygenase inhibitors, such as nonsteroid anti-inflammatory agents (NSAIDs), also exhibit (weak and reversible) antiplatelet effects, but this effect is less clinically relevant and never requires immediate reversal.

Thienopyridine derivatives (including clopidogrel, prasugrel, or cangrelor) act by blocking the adenosine diphosphate (ADP) receptor on the platelet (see Chap. 18). Clinical studies have shown that these agents are as good as aspirin in the secondary prevention of atherothrombotic events. Importantly, the combination of aspirin and clopidogrel is vastly superior over aspirin alone in patients who have recent or drug-eluting intracoronary stents or in other patients with high-risk coronary artery disease. However, the increased efficacy of the combined use of aspirin and clopidogrel is also associated with a significantly higher bleeding risk [16]. The decision whether or not the interrupt or even reverse antithrombotic treatment with dual platelet inhibition in case of serious bleeding or the need to perform an invasive procedure will depend on the specific clinical situation but also on the indication for the antithrombotic treatment. Especially in patients with recent implantation of an intracoronary stent (in the last 6–12 weeks) or with drug-eluting stents, there is a high risk of thrombotic stent occlusion associated with cessation of treatment [17]. Other agents that impact platelet function are glycoprotein IIb/IIIa antagonists (such as abciximab, eptifibatide, or tirofiban), which are usually applied in the setting of coronary angioplasty (see Chap. 18). In addition, selective serotonin release inhibitors (SSRI's) have been associated with impaired platelet function, mostly when combined with other agents affecting primary hemostasis or in patients with other coagulation defects.

Acquired Impaired Fibrin Formation

Acquired Coagulation Factor Deficiency

Table 13.1 summarizes the various acquired causes of impaired fibrin formation. Low concentrations of coagulation factors may be the result of impaired synthesis, increased loss, or enhanced and insufficiently compensated consumption.

As all coagulation factors (except factor VIII) are synthesized by the liver, severe liver dysfunction may result in decreased coagulation factor levels. This fact is reflected in prolonged global coagulation times (aPTT and PT) and may contribute to an enhanced bleeding tendency.

Vitamin K plays an essential role in the synthesis of several coagulation factors. The vitamin K-dependent coagulation factors (factors II (prothrombin), VII, IX, and X) undergo vitamin K-dependent posttranslational carboxylation in the liver prior to secretion into plasma. This step allows the coagulation factors to become active by attaining the ability to bind to calcium. Hence, in the absence of vitamin K, inactive precursor molecules are formed. Vitamin K is normally present in food (e.g., green vegetables and cabbage) and is produced by bacteria in the gut. Hence, vitamin K deficiency may be caused by insufficient food intake or malabsorption (e.g., due to the absence of bile salts that enable the uptake of the fat-soluble vitamin K) or can be due to significant changes in the intestinal bacterial flora.

Uncompensated loss of coagulation factors may occur after massive bleeding, as in trauma patients or patients undergoing major surgical procedures. This event is particularly common in patients with major blood loss where intravascular volume is rapidly replaced with crystalloids, colloids and red cells without simultaneous administration of coagulation factors [18]. Infusion of large volumes of plasma expanders may have a direct detrimental effect on coagulation by itself [19]. In hypothermic patients (e.g., trauma patients), measurement of the global coagulation tests may underestimate coagulation in vivo, since in the laboratory test tube assays are standardized and performed at 37 °C.

Fresh frozen plasma contains all coagulation factors and may be used to replenish deficiencies of these clotting factors. Most consensus guidelines indicate that plasma should only be transfused in case of bleeding, or if a high-risk of bleeding exists, and not based on laboratory abnormalities alone. For more specific therapy or if the transfusion of large volumes of plasma is not desirable, fractionated plasma of purified coagulation factor concentrate (such as prothrombin complex concentrate, containing all vitamin K-dependent coagulation factors, or fibrinogen concentrate) is available. Vitamin K deficiency can simply be treated by administering vitamin K (see further).

Coagulation Factor Inhibitors

Clinically significant autoantibodies to coagulation factors are rare but can result in life-threatening bleeding and death. The most commonly targeted coagulation factor by an autoantibody is factor VIII (acquired hemophilia A) (see Chap. 12), but also other coagulation factors, such as factor V, may be inhibited by an autoantibody. Acquired hemophilia A can either be idiopathic or associated with other autoimmune disorders, malignancy, postpartum period, and the use of drugs (such as antibiotics) [20]. The incidence of autoantibodies to factor VIII is 0.2–1 per 1 million persons per year. Patients with acquired hemophilia A usually pres-

ent with spontaneous bleeding, which can be severe and life-threatening. Common bleeding sites are soft tissues, skin, and mucous membranes. In contrast to patients with congenital hemophilia A (and poorly understood), hemarthroses, muscle bleeding, and central nervous system hemorrhage are uncommon. Once the identity of an inhibitor has been established by mixing studies (see above), the antibody titer is determined using the Bethesda assay. The inhibitor titer is defined as the dilution of patient plasma that produces 50% inhibition of the factor VIII activity and is expressed as Bethesda units per mL (BU/mL). Inhibitors are classified as low titer or high titer when the titers are less than 5 BU/mL or greater than 5 BU/mL, respectively. Acquired factor VIII inhibitors sometimes resolve spontaneously. However, it is not possible to predict in which subset of patients this will occur, and so treatment will be required when bleeding complications ensue.

Patients with a factor VIII inhibitor titer of less than 5 BU/mL often are treated successfully with sufficient doses of recombinant or plasma-derived factor VIII concentrates to neutralize the inhibitor. Patients with titers between 5 and 10 BU/mL also may respond to factor VIII concentrates, whereas those with titers greater than 10 BU/mL generally do not respond. Factor VIII bypassing agents are used to manage patients with a high-titer inhibitor. Two agents, recombinant-activated factor VII and activated prothrombin complex concentrate (also called factor eight inhibitor bypassing agent or FEIBA), are licensed for treatment of acquired hemophilia A. Recently, a bispecific monoclonal antibody called emicizumab has been approved for treatment of FVIII inhibitors. This monoclonal antibody binds factors IX/IXa and X/Xa to substitute for FVIII. Although acquired inhibitors may remit spontaneously, initiation of immunosuppressive treatment at the time of diagnosis to eradicate the inhibiting antibody is recommended because of the severe course of this disorder. A variety of immunosuppressive agents have been used, including prednisone, cyclophosphamide, azathioprine, and rituximab. Plasmapheresis and immunoglobulin immunoadsorption of the inhibitory antibody have been advocated, but they are temporizing procedures at best. Also, immune tolerance induction using high-dose factor VIII has been used successfully.

Antibodies inhibiting thrombin and factor V frequently coexist in immune responses to commercial products that contain (bovine) coagulation factors (e.g., adhesive tissue glue) [21]. Some of these products are contaminated with other plasma proteins, including factor V and prothrombin. Almost all patients exposed to bovine proteins develop a detectable immune response. In half of these patients, anti-bovine antibodies cross-react with human thrombin, factor V, or prothrombin. Normally, these antibodies cause no clinical problems. However, mild to life-threatening hemorrhage can occur, especially if the titer of antihuman factor V antibodies is high. Beta-lactam antibiotics have also been associated with anti-factor V autoantibodies, and anti-factor V autoantibodies have been identified rarely in patients with autoimmune diseases, solid tumors, and monoclonal gammopathies. Management of bleeding complications and the underlying antibody follows similar principles as treatment of acquired hemophilia.

Bleeding Due to Anticoagulants

Anticoagulant agents are often used for prevention and treatment of a wide range of cardiovascular diseases. Most frequently used anticoagulants are heparin or its derivatives, or vitamin K antagonists (such as warfarin or Coumadin), and a few years ago, a new class of oral direct-acting anti-factor Xa or anti-factor IIa (thrombin) agents [direct oral anticoagulants (DOACs)] have been introduced. The most important complication of treatment with anticoagulants is hemorrhage, which may be serious, may cause long-term debilitating disease, or may even be life-threatening [22]. If severe bleeding occurs or if a patient needs to undergo an urgent invasive procedure, such as emergency surgery, it may be required to reverse the anticoagulant effect of the various agents. Depending on the clinical situation, i.e., the severity of the bleeding or the urgency and estimated risk of the invasive procedure, this reversal may take place in a few hours, but in some cases immediate reversal is necessary. Generally, each (immediate) reversal of anticoagulant treatment needs also to take into consideration the indication for the antithrombotic agents. For example, in a patient with a prosthetic mitral valve and atrial fibrillation, interruption of vitamin K antagonists may increase the risk of valve thrombosis and cerebral or systemic embolism. Each of these specific clinical situations requires a careful and balanced assessment of the benefits and risks of reversing anticoagulants (and potential strategies to keep the period of reversal as short as possible) [23].

Heparin and heparin derivatives act by binding to antithrombin and thereby about 1000-fold potentiating the anticoagulant effect of this endogenous inhibitor toward thrombin and factor Xa (and some other coagulation factors). Unfractionated heparin causes a prolongation of the aPTT; however, low molecular weight (LMW) heparin has no effect on the aPTT. The anti-factor Xa assay is the best test to quantitate plasma levels of both unfractionated heparin and LMW heparin. Heparin has a relatively short half-life of about 60–90 min, and therefore the anticoagulant effect of therapeutic doses of heparin will be mostly eliminated at 3–4 h after termination of continuous intravenous administration [24]. The anticoagulant effect of high-dose subcutaneous heparin, however, will take a longer time to abolish. If a more immediate neutralization of heparin is

required, intravenous protamine sulfate is the antidote of choice. The reversal of LMW heparin is more complex, as protamine sulfate will only neutralize the anti-factor IIa activity and has no or only partial effect on the smaller heparin fragments causing the anti-factor Xa activity of the compound. Pentasaccharides are synthetic compounds that effectively bind and potentiate antithrombin to block factor Xa. Since they lack the additional glycosaminoglycan saccharide residues to bind to thrombin, it has an effect on factor Xa exclusively. The prototype pentasaccharide (and the only one approved for clinical use so far) is fondaparinux. The only agent that has been systematically evaluated to reverse the anticoagulant effect of pentasaccharides is recombinant factor VIIa (rVIIa). And although there are no controlled trials in patients who present with pentasaccharide-induced bleeding, there is some anecdotal experience suggesting that rVIIa may indeed be able to stop bleeding in patients anticoagulated with fondaparinux. Similarly, prothrombin complex concentrates (PCCs) and andexanet alpha have anti-factor Xa neutralizing ability as well. Neither of these agents presently have regulatory approval for their use.

Treatment with vitamin K antagonists (VKAs) (such as warfarin, Coumadin, acenocoumarol, or phenprocoumon) increases the risk of major bleeding by 2–3%/year and the risk of intracranial hemorrhage by about 0.3–0.5%/year [23]. The most important risk factor for hemorrhage in users of VKAs is the intensity of the anticoagulant effect. Studies indicate that with a target INR of >3.0, the incidence of major bleeding is twice as large as in studies with a target INR of 2.0–3.0. A retrospective analysis of outpatients using warfarin who presented with intracranial hemorrhage demonstrated that the risk of this complication doubled for each one unit increment of the INR [25]. Patient characteristics constitute another important determinant of the bleeding risk. Elderly patients have a twofold increased risk of bleeding, and the relative risk of intracranial hemorrhage (in particular at higher INR's) was 2.5 (95% CI 2.3–9.4) in patients >85 years compared to patients 70–74 years old. Genetic factors, such as common polymorphisms in the P450 CYP2C9 enzyme or variants in the vitamin K epoxide reductase complex subunit 1 gene (*VKORC1*), may be associated with a higher risk of bleeding. Comorbidity, such as renal or hepatic insufficiency, may also significantly increase the risk of hemorrhagic complications. The combined use of VKAs and NSAIDs may result in an 11-fold higher risk of hospitalization for gastrointestinal bleeding as compared to the general population [26]. The intensity of anticoagulation with VKAs is assessed with the prothrombin time or its internationally standardized variant the INR (see Chap. 11). In case of major bleeding, it may be required to reverse the anticoagulant effect of VKAs [27]. When interrupting the administration of VKAs important differences in the half-lives of the various agents (9 h for acenocoumarol, 36–42 h for warfarin, and 90 h for phenprocoumon, respectively) need to be taken into account [28]. The most straightforward intervention to counteract the effect of VKAs is the administration of vitamin K [29]. There is quite some debate on the use of vitamin K in patients with a too high INR but no signs of bleeding. However, a randomized controlled trial did not find any difference in bleeding or other complications in nonbleeding patients with INR values of 4.5–10 that were treated with vitamin K or placebo [30]. In patients with clinically significant bleeding, administration of vitamin K is crucial to reverse the anticoagulant effect of VKAs. Vitamin K (2–5 mg) can be given orally and intravenously, whereas the parenteral route has the advantage of a more rapid onset of the treatment [31]. A potential concern with the use of parenteral vitamin K is the occurrence of anaphylactic reactions, although the incidence of this complication is very low, in particular with the more modern micelle preparations [32]. In case of very serious or even life-threatening bleeding, immediate correction of the INR is mandatory and can be achieved by the administration of vitamin K-dependent coagulation factors. Theoretically, these factors are present in (fresh frozen) plasma; however, the amount of plasma that is required to correct the INR is very large, carries the risk of fluid overload, and will probably take hours to administer [33]. Therefore, prothrombin complex concentrates (PCCs), containing all vitamin K-dependent coagulation factors, are more useful. In a prospective study in patients using VKA and presenting with bleeding, PCCs resulted in at least satisfactory and sustained hemostasis in 98% of patients [34].

DOACs (rivaroxaban, apixaban, edoxaban, betrixiban, dabigatran) are associated with similar or slightly lower major hemorrhagic complications compared with vitamin K antagonists [35]. Rivaroxaban, apixaban, edoxaban, and betrixiban are directed to factor Xa; dabigatran is directed to factor IIa (thrombin) (see Chap. 17). Of note, DOAC treatment is associated with a reduced intracranial hemorrhage incidence compared with warfarin therapy, whereas some analyses showed an increased risk for gastrointestinal bleeds. The concentration of direct oral anti-Xa agents in plasma can be assessed by the prothrombin time or by measuring anti-factor Xa levels. Monitoring anti-IIa levels requires an ecarin clotting time or establishment of an anti-factor IIa chromogenic assay, which is not readily available in most clinical settings. Dependent on the severity of the clinical situation and in view of the relatively short half-life of the DOACs (5–15 h), cessation of medication may often be sufficient to reverse the anticoagulant effect in case of bleeding. It is likely that in most cases, this will suffice, and more immediate reversal is hardly ever really needed in clinical practice [36]. However, if immediate reversal of anticoagulation is deemed necessary (i.e., intracerebral hemorrhage), additional measures may be required. In general, these can be divided in non-specific and specific interventions to reverse the anti-

coagulant effect. Non-specific measures include prothrombin complex concentrates or recombinant factor VIIa (rFVIIa) that was shown to reverse the anticoagulant effect of direct-acting Xa inhibitors but is probably less effective in reversing anti-IIa agents. Because the prothrombotic potential of rFVIIa might be higher than that of non-activated PCCs, non-activated PCCs may be preferred [37]. Specific measures to reverse the DOACs include new agents such as a recombinant protein analog of factor Xa that binds to factor Xa inhibitors but does not trigger prothrombotic activity (andexanet). This agent is still in clinical trials. Dabigatran is reversed by an approved agent that is a Fab fragment of a monoclonal antibody (idarucizumab) directly binding to dabigatran and eliminating its anticoagulant effect [38, 39]. Both these agents were shown to virtually immediately reverse the anticoagulant activity of direct oral anti-Xa or anti-IIa agents, respectively.

Consumption Coagulopathy

A variety of disorders, including infectious or inflammatory conditions and malignant disease, will lead to activation of coagulation. In many cases, this activation of coagulation will not lead to clinical complications and will not even be detected by routine laboratory tests, but can only be measured with sensitive molecular markers for activation of coagulation factors and pathways [40]. However, if activation of coagulation is sufficiently strong, the platelet count may decrease, and global clotting times may become prolonged. In its most extreme form, systemic activation of coagulation is known as disseminated intravascular coagulation (DIC). DIC is characterized by the simultaneous occurrence of widespread (micro)vascular thrombosis, thereby compromising blood supply to various organs, which may contribute to organ failure [41]. Because of ongoing activation of the coagulation system and other factors, such as impaired synthesis and increased degradation of coagulation proteins and protease inhibitors, consumption of clotting factors and platelets may occur, resulting in bleeding from various sites. DIC is not a disease in itself but can only occur secondary to a number of distinct clinical entities (Table 13.4).

Figure 13.2 schematically depicts the pathogenesis of DIC. The most important initiator of thrombin formation in DIC is tissue factor. Studies of experimental or human endotoxemia or cytokinemia have demonstrated a central role of the tissue factor/factor VIIa system in the initiation of thrombin generation [42]. In addition, platelets have a central role in the development of coagulation abnormalities in DIC. Platelets can be triggered directly by pro-inflammatory mediators, and generated thrombin will further activate platelets. The expression of P-selectin on the platelet membrane not only mediates the adherence of platelets to leukocytes and endothelial cells but also enhances the expression of tissue factor on monocytes. In DIC all three important physiological anticoagulant pathways (the antithrombin system, the activated protein C system, and tissue factor pathway inhibitor (TFPI)) are importantly impaired [43]. Due to a combination of impaired synthesis, ongoing consumption and proteolytic degradation (e.g., by neutrophil elastase) levels of all three coagulation inhibitors are low. Also, significant downregulation of thrombomodulin and endothelial protein C receptor (EPCR) in DIC will cause reduced conversion of protein C to activated protein C. In addition, at the time of the greatest activation of coagulation in DIC, endogenous fibrinolysis is largely turned off. After an initial acute release of plasminogen activators (i.e., tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA)) from storage sites in vascular endothelial cells, the increase in plasminogen activation and subsequent plasmin generation is annihilated by a sustained increase in plasminogen activator inhibitor, type 1 (PAI-1).

Table 13.4 Most frequent underlying clinical conditions that can be complicated by DIC

Sepsis/severe infection
Trauma/burn/heatstroke
Malignancy
Solid tumors
Acute leukemia
Obstetrical conditions
Amniotic fluid embolism
Abruptio placentae
HELLP syndrome
Vascular abnormalities
Kasabach-Merritt syndrome
Other vascular malformations
Aortic aneurysms
Severe allergic/toxic reactions
Severe immunologic reactions (e.g., transfusion reaction)

Patients with DIC have a low or rapidly decreasing platelet count, prolonged coagulation tests, low plasma levels of coagulation factors and inhibitors, and increased markers of fibrin formation and/or degradation, such as D-dimer or fibrin degradation products (FDPs). A diagnosis of DIC may be made using a simple scoring system (Table 13.5) based on a combination of routinely available coagulation tests (platelet count, prothrombin time, D-dimer levels, and fibrinogen) [44]. The sensitivity and specificity of this DIC score were found to be 93% and 98%, respectively [45]. Furthermore, this DIC score was a strong and independent predictor of mortality in a large series of patients with severe sepsis.

The foundation of the treatment of DIC is the management of the underlying disorder, e.g., by appropriate antibiotics or cancer treatment. However, coagulation may need supportive measures as the coagulopathy may proceed even after adequate treatment has been initiated. Low levels of

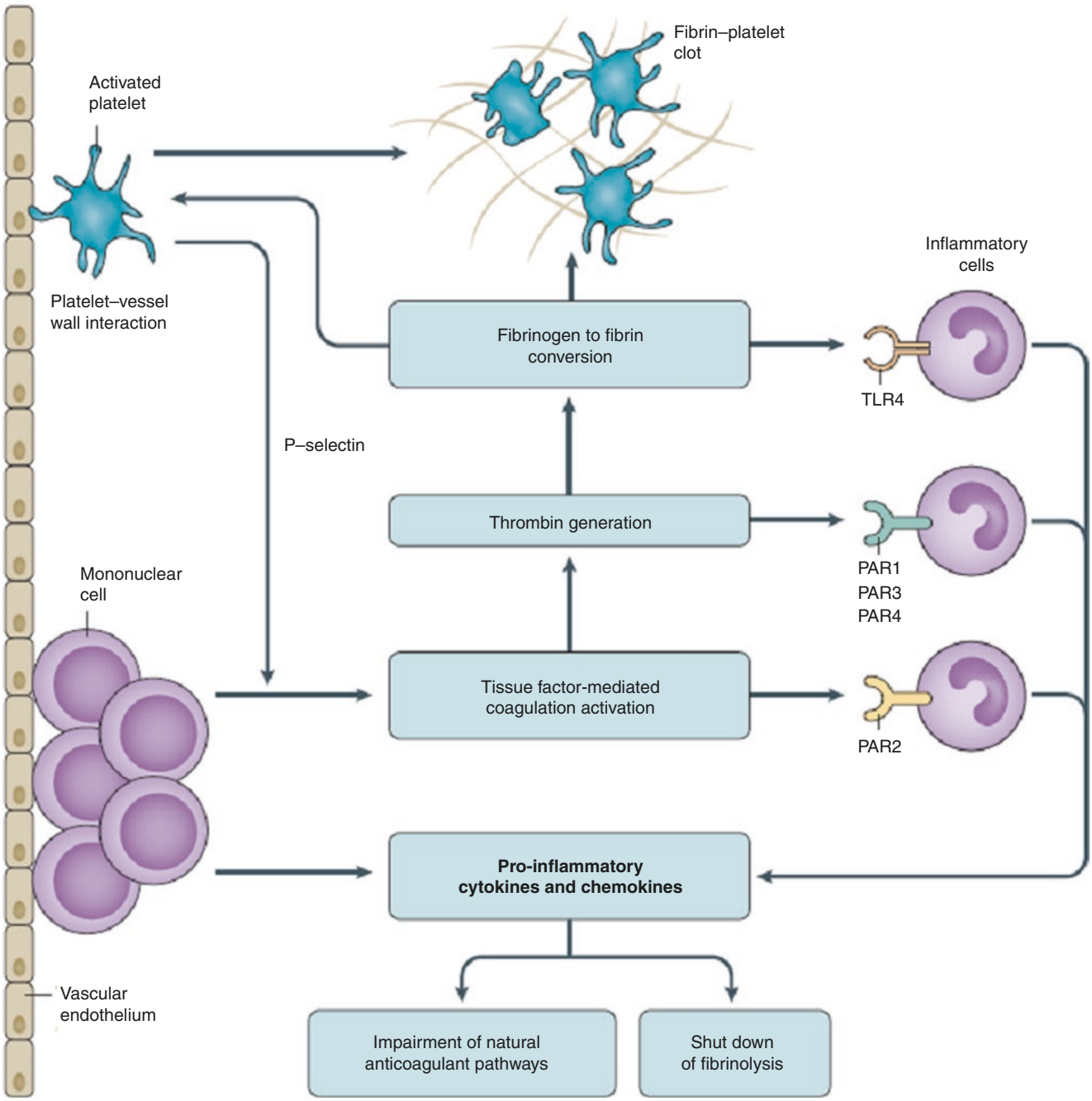

Fig. 13.2 Pathogenesis of DIC. Expression of tissue factor by mononuclear cells and subsequent exposure to blood results in the generation of thrombin followed by the conversion of fibrinogen to fibrin. Platelet-derived P-selectin further enhances the expression of tissue factor. The binding of tissue factor, thrombin, and other activated coagulant proteases to specific protease-activated receptors (PARs) and the binding of fibrin to Toll-like receptor 4 (TLR4) on inflammatory cells affect inflammation through the consequent release of pro-inflammatory cytokines and chemokines, which further modulates coagulation and fibrinolysis

platelets and coagulation factors may increase the risk of bleeding. However, plasma or platelet substitution therapy should not be instituted on the basis of laboratory results alone; it is indicated only in patients with active bleeding and in those requiring an invasive procedure or otherwise at risk for bleeding complications [6]. Experimental studies have shown that heparin partly inhibits the activation of coagulation in sepsis. However, an advantageous effect of heparin on clinically important outcome events in patients with DIC has never been clearly demonstrated in controlled clinical trials, although there is cumulating evidence that heparin is beneficial [46, 47]. In addition, there are several studies showing that critically ill patients with DIC need adequate prophylaxis for venous thromboembolism, usually with (LMW)

Table 13.5 Diagnostic algorithm for the diagnosis of overt DIC

1. Presence of an underlying disorder known to be associated with DIC
(no = 0, yes = 2)
If no: do not proceed with this algorithm
2. Score global coagulation test results
Platelet count (*>100 = 0; <100 = 1; <50 = 2*)
Level of fibrin markers (e.g., D-dimer, fibrin degradation products)
(*no increase: 0; moderate increase: 2; strong increase: 3*)[a]
Prolonged prothrombin time
(*<3 s = 0; >3 s but <6 s = 1; >6 s = 2*)
Fibrinogen level
(*>1.0 g/L = 0; <1.0 g/L = 1*)
3. Calculate score
4. If ≥5: compatible with overt DIC
If <5: suggestive (not affirmative) for non-overt DIC; repeat next 1–2 days

According to the Scientific Standardization Committee of the International Society of Thrombosis and Hemostasis

[a]Strong increase >5× upper limit of normal; moderate increase is > upper limit of normal but <5× upper limit of normal

heparin [48]. Restoration of the levels of physiological anticoagulants in DIC may be a rational approach. All trials with administration of these anticoagulant concentrates, such as antithrombin concentrate or recombinant-activated protein C, have shown some beneficial effect in terms of improvement of laboratory parameters, or shortening of the duration of the coagulopathy, but large-scale, multicenter, randomized controlled trials, mostly in sepsis, did not show a significant reduction in mortality of patients with DIC. The most promising intervention at this moment is recombinant soluble thrombomodulin. In initial clinical trials in patients with DIC, administration of the soluble thrombomodulin had a significantly better effect on bleeding manifestations and organ dysfunction than heparin, and a lower mortality. The promising results with recombinant soluble thrombomodulin are supported by retrospective data in large series of Japanese patients and are currently being evaluated in large international multicenter trials [49].

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Platelet Function in Hemostasis and Inherited Disorders of Platelet Number and Function

14

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Platelet Structure

Blood platelets are anucleate fragments derived from bone marrow megakaryocytes. They are 1.5–3.0 μm in diameter with a volume of ~7 fl. Electron microscopy reveals a fuzzy coat (glycocalyx) on the platelet surface composed of membrane glycoproteins (GP), glycolipids, mucopolysaccharides, and plasma proteins. The plasma membrane is a bilayer of phospholipids in which cholesterol, glycolipids, and glycoproteins are embedded. Platelets have an elaborate channel system, the open canalicular system, which is composed of invaginations of the plasma membrane. In addition, they have a dense tubular system, a closed-channel network derived from the smooth endoplasmic reticulum; it is the major site of platelet thromboxane synthesis. The discoid shape of resting platelets is maintained by a cytoskeleton consisting of a spectrin membrane, a microtubule coil, and an actin scaffold.

Platelets contain several organelles: mitochondria and glycogen stores, lysosomes, dense (δ) granules, and α granules. The dense granules contain calcium, ATP, ADP, magnesium, serotonin, and polyphosphates. Serotonin is not synthesized by platelets or megakaryocytes but is incorporated from ambient plasma. The α granules contain numerous proteins, including β-thromboglobulin (βTG) and platelet factor 4 (PF4), which are considered platelet-specific, several coagulation factors, (e.g., fibrinogen, factor V, high molecular weight kininogen, factor XIII), von Willebrand factor (vWF), growth factors (e.g., platelet-derived growth factor, vascular endothelial growth factor), protease inhibitors (e.g., plasminogen activator inhibitor-1, C1 inhibitor, amyloid-β-protein precursor), thrombospondin, P-selectin, albumin, and IgG. Several of these proteins (e.g., albumin, fibrinogen, factor V, IgG) are not synthesized by human platelets or megakaryocytes but are incorporated by endocytosis. The lysosomes contain acid hydrolases and other enzymes. Granule constituents are secreted on platelet activation, and this is dependent on the strength of activation and differs between agonists.

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Platelet Function in Hemostasis

Adhesion Following injury to the blood vessel, platelets adhere to exposed subendothelial collagen (adhesion), which involves among other events the interaction of a plasma protein, von Willebrand factor (vWF), and a specific glycoprotein complex on the platelet surface, glycoprotein (GP) Ib-IX-V (Fig. 14.1). This interaction is particularly important for platelet adhesion under conditions of high shear stress (i.e., high blood flow). Glycoprotein VI also contributes to platelet interaction with collagen under conditions of flow.

Aggregation Adhesion is followed by recruitment of additional platelets which form clumps, a process of platelet-platelet interaction called aggregation. This involves binding of fibrinogen to specific platelet surface receptors – a complex comprised of glycoproteins IIb and IIIa (GPIIb-IIIa, integrin $\alpha_{2b}\beta_3$) (Fig. 14.1). Binding of fibrinogen to platelets is a prerequisite for aggregation in response to all physiologic agonists. On platelet activation, the GPIIb-IIIa complex undergoes a conformational change and acquires the ability to bind fibrinogen; resting platelets do not bind fibrinogen.

Secretion (Also Called Release Reaction) Activated platelets release contents of their granules including from dense granules, α-granules, and the lysosomal vesicles. ADP and serotonin released from the dense granules interact with specific receptors on platelets to enhance the activation process.

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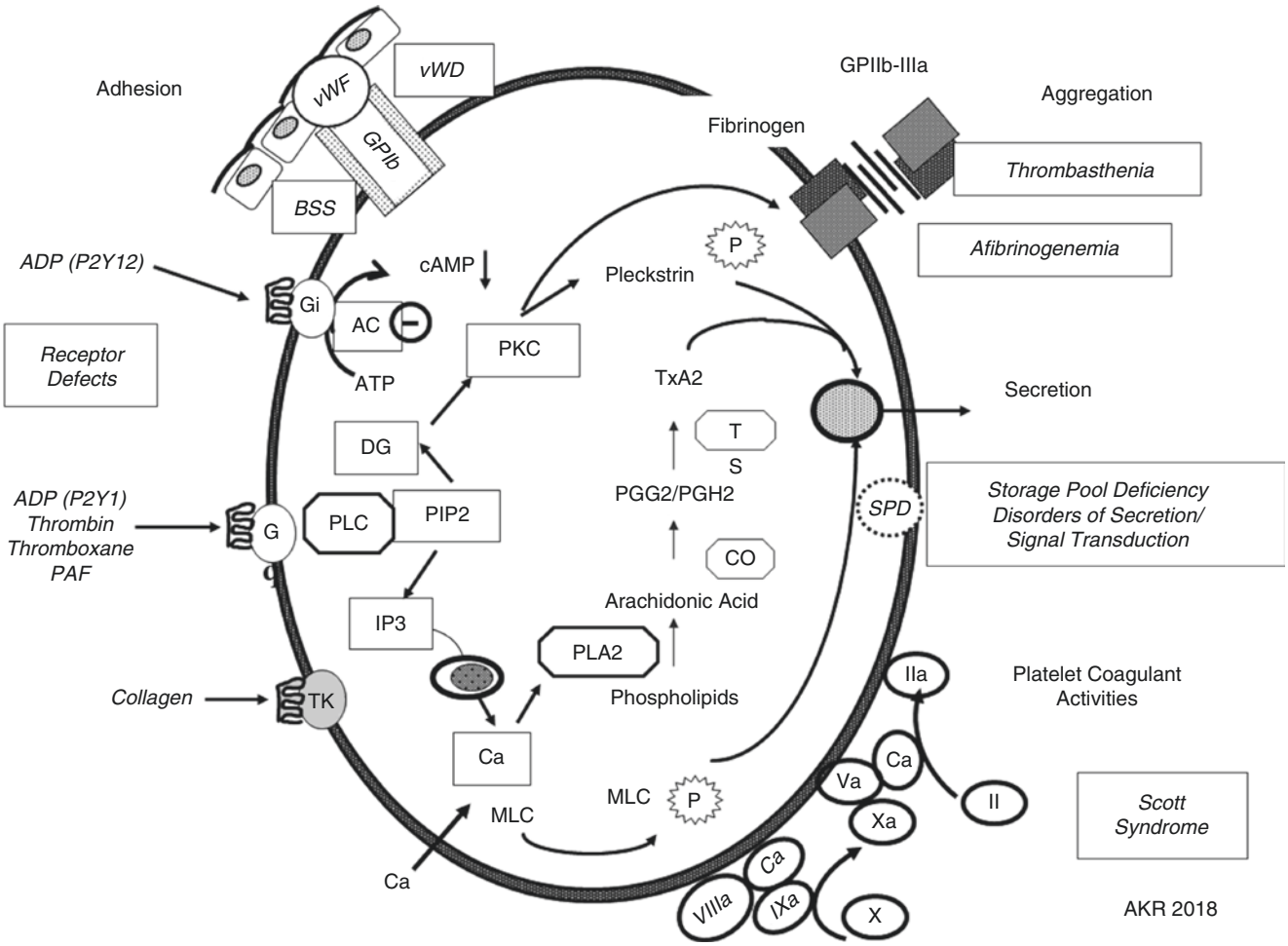

Fig. 14.1 Schematic representation of selected aspects of platelet responses to activation and inherited disorders of platelet function. Following vessel wall injury, platelets adhere to subendothelium, and this is mediated by binding of vWF to platelet GPIb (left upper section of figure). Platelet aggregation is mediated by the interaction of fibrinogen with the GPIIb-IIIa complex on platelet surface (right upper corner). Platelets possess receptors for several agonists, such as ADP, thrombin, thromboxane A_2, and collagen. Receptor activation results in the formation of intracellular mediators that regulate the end responses, such as aggregation and secretion. Activation of intracellular enzymes on receptor activation is mediated by GTP-binding proteins (G). Responses to collagen are mediated by tyrosine kinase (TK)-dependent mechanisms. Receptor activation leads to hydrolysis of phosphatidylinositol bisphosphate (PIP_2) to form diacylglycerol (DAG), which activates protein kinase C (PKC), and inositol trisphosphate (IP_3), which mediates the rise in cytoplasmic Ca^{2+} levels. This increase in Ca^{2+} levels leads to other responses, such as activation of myosin light chain kinase (MYLK) to phosphorylate myosin light chain (MLC) and activation of phospholipase A_2 (PLA_2), which mediates the release of free arachidonic acid from phospholipids. This arachidonic acid is converted by cyclooxygenase (CO) and thromboxane synthase (TS) to thromboxane A_2. As a result of several of these mechanisms, platelets release granule contents (secretion). Moreover, platelets play a major role in blood coagulation mechanisms by providing the membrane surface on which several of the key reactions occur (platelet coagulant activities). The coagulation factors are shown by Roman numerals in circles. The figure also shows some of the inherited disorders of platelet function. *Disorders of adhesion* arise due to defects/deficiency in GPIb complex (Bernard-Soulier syndrome) or in plasma vWF (von Willebrand disease). *Disorders of aggregation* arise due to defects in GPIIb-IIIa on platelets or the absence of plasma fibrinogen (right upper corner). *Defects in platelet activation mechanisms and secretion* may occur secondary deficiencies of platelet surface receptors (receptor defects) and defects at the level of G-proteins and in other signal transduction events (described in the text). In *storage pool deficiency*, secretion is impaired because the granules or their contents are decreased. Several patients have been described with *defects in thromboxane A2 synthesis* due to deficiencies of phospholipase A2, cyclooxygenase, or thromboxane synthase. In the Scott syndrome the contribution of platelets to the coagulation system is impaired. (Modified with permission from The American Journal of the Medical Sciences, 316, Rao, AK, Congenital Disorders of Platelet Function: Disorders of Signal Transduction and Secretion, 69–76, Elsevier, 1998)

Platelet Procoagulant Activities Several key enzymatic reactions of blood coagulation occur on the platelet membrane lipoprotein surface. During platelet activation, negatively charged phospholipids, especially phosphatidylserine, translocate from the inner aspect of the plasma membrane to the platelet surface (Fig. 14.1). This translocation is an essential step in accelerating specific coagulation reactions that occur on the platelet surface and lead to thrombin generation.

Platelet Activation Mechanisms

A number of physiological agonists interact with receptors on the platelet surface to induce responses, including transmission of the signal from the surface receptors (signal transduction), formation of intracellular mediators, and culminating in a change in platelet shape from discoid to spherical, platelet aggregation, secretion, and thromboxane A2 (TxA_2) production. Selected aspects of platelet activation are shown in Fig. 14.1. Activation of platelet surface receptors initiates the production of several intracellular messenger molecules, including products of hydrolysis of phosphoinositide by phospholipase C (diacylglycerol and inositol 1,4,5-triphosphate), TxA_2, and cyclic nucleotides (cAMP) (Fig. 14.1). These induce or modulate the various platelet responses such as the rise in cytoplasmic Ca2+ concentration, protein phosphorylation, aggregation, secretion, and TxA_2 production. The interaction between the platelet surface receptors and the key intracellular enzymes (e.g., phospholipases A2 and C, adenylyl cyclase) is mediated by a group of GTP-binding proteins (G-proteins) that function as molecular switches. Platelet activation results in a rise in cytoplasmic ionized calcium concentration. InsP3 functions as a messenger to mobilize Ca2+ from intracellular stores. Diacylglycerol activates protein kinase C (PKC) resulting in the phosphorylation of several proteins. PKC activation plays a major role in platelet responses, including secretion and in the activation of GPIIb-IIIa. GTP-binding protein Gαq mediates the activation of phospholipase C-β2 on activation of platelet $P2Y_1$-ADP, thrombin, thromboxane, and other receptors. Gαi mediates the inhibition of adenylyl cyclase on platelet activation with $P2Y_{12}$-ADP receptor and other receptors. Lastly, Gαs stimulates adenylyl cyclase to increase cAMP levels in platelets; increased intracellular cAMP levels inhibit platelet responses and play a role in maintaining platelets in the quiescent resting state as they circulate in blood. Numerous other mechanisms, such as activation of tyrosine kinases and phosphatases, are also triggered by platelet activation and are reviewed elsewhere [1].

Regulation of Platelet Number

Platelets are produced from bone marrow megakaryocytes; approximately 1×10^{11} platelets are produced daily. The normal platelet count is 150,000–400,000/μl. Platelets have a lifespan half-life in blood of 7–10 days. The number of circulating platelets is regulated by thrombopoietin (TPO), which is synthesized in the liver and binds to megakaryocytes and hematopoietic stem cells via the TPO receptor, c-Mpl. Because of this binding, there is an inverse relationship between platelet mass and circulating TPO levels.

Inherited Disorders of Platelets

Two categories of inherited platelets disorders are recognized: disorders associated with decreased platelet number (thrombocytopenia) and those characterized by impaired platelet function. Some patients may have a combination of inherited thrombocytopenia and platelet dysfunction. Patients with platelet disorders often have excessive bleeding involving the skin or mucus membranes. This can manifest clinically with symptoms and signs such as easy bruising, epistaxis, gum bleeding, menorrhagia, or petechiae. As platelets are the major player in primary hemostasis, onset of bleeding after a hemostatic challenge such as trauma or surgery is generally immediate. There can also be a family history of bleeding, and an inheritance pattern can be suggested.

Evaluation of Platelet Number and Function

Platelet Count and Size and Peripheral Blood Smear The evaluation of patients with inherited platelet disorders starts with a complete blood count that includes platelet count and examination of the peripheral smear to detect alterations in platelet morphology. If the platelet count is normal, approximately 8–15 platelets are visible in each oil-immersion (X1000) field; one platelet is observed for every 20 erythrocytes.

Bleeding Time Although this test is no longer available in most centers, it is worthy of mention because of the important information it has provided in our recognition of patients with platelet disorders. The bleeding time test involves making an incision on the volar surface of the forearm under standardized conditions (i.e., with a blood pressure cuff inflated to 40 mm Hg) and monitoring the time it takes for the bleeding to stop (normal range is 2–8 min). It is prolonged in many patients with thrombocytopenia (with counts less than ~70,000/ul) and those with impaired platelet function (inherited or acquired). It is also prolonged in disorders affecting the vessel wall, such as connective tissue disorders (e.g., pseudoxanthoma elasticum) and scurvy, as well as disorders involving abnormalities in the interaction of platelets with the subendothelium (e.g., von Willebrand disease). The limitations of the bleeding time are that it is labor-intensive, is operator dependent, and may lead to scarring at the incision site.

In Vitro Studies of Platelet Function Several tests have been developed that reflect the function of platelets in vitro [2, 3]. A widely used method to assess platelet aggregation and secretion is performed using platelet-rich plasma (PRP) prepared from blood. Light transmission through a glass cuvette containing PRP is monitored in an instrument called the aggregometer. On addition of an agonist (such as ADP, colla-

gen, epinephrine, arachidonic acid, or ristocetin), the platelets in the cuvette form clumps thereby increasing the light transmission, which is recorded. In response to an agonist, such as ADP or epinephrine, normal platelets show an initial increase in light transmission, called the primary wave of aggregation, which is followed by release of dense granule contents and thromboxane production leading to a second, irreversible wave of aggregation. With agonists such as thrombin, collagen, and arachidonic acid, only one large wave of aggregation is generally discerned without a distinct primary and secondary wave. Platelet dense granule secretion can also be simultaneously measured in this system by monitoring the release of ATP using an instrument called the lumi-aggregometer or by using radioactive 5-hydroxytryptamine (serotonin), which is incorporated by platelets into the dense granules and released upon platelet activation [2]. Studies performed with a platelet aggregometer can be corroborated with studies on agonist-mediated platelet activation using flow cytometry-based approaches. This technique uses less platelets and has the potential to provide additional information.

Another method to test platelet function in vitro, called the platelet function analyzer (PFA-100), measures platelet adherence under shear by passing whole blood through an aperture in a membrane coated with collagen in combination with ADP or epinephrine [2]. The time to closure of the aperture is measured. In many disorders of platelet function, the closure time is prolonged, but PFA is neither sensitive nor specific to detect inherited defects in platelets [4]. The PFA closure time is prolonged in VWD as well.

Inherited Thrombocytopenias

The clinical spectrum of inherited thrombocytopenias ranges from severe bleeding starting early in life to being asymptomatic into adulthood. Often the major diagnostic distinction in a patient with thrombocytopenia is between inherited thrombocytopenia and immune thrombocytopenia. Therefore, attaining information on previous platelet counts is vital in the evaluation of the patient with thrombocytopenia. Patients with inherited thrombocytopenias may have a family history of thrombocytopenia or other hematologic disorders.

It is important to distinguish between inherited thrombocytopenia and immune thrombocytopenia for several reasons. This includes preventing unnecessary exposure to treatments used for immune thrombocytopenia, such as steroids, intravenous immunoglobulins, and splenectomy. Diagnosis of inherited thrombocytopenia can also have prognostic impact or counseling implications, such as the predisposition to myeloid malignancies in patients with inherited thrombocytopenia from *RUNX1* and *ANKRD26* germline mutations, or hearing loss or renal dysfunction in patients with *MYH9* mutations. Correct diagnosis of an inherited thrombocytopenia also informs treatment, as the thrombopoietin agonist eltrombopag has been used to augment platelet count in patients with *MYH9* mutations [5]. Lastly, some patients with inherited thrombocytopenia also have platelet function abnormalities, as in patients with *RUNX1* mutations [6]. Such patients may have severity of bleeding symptoms out of proportion to their platelet count and require additional treatment considerations.

Inherited thrombocytopenias are classified by the inheritance pattern, clinical features (age of presentation or presence of associated non-platelet abnormalities), or platelet size. Platelet size on the peripheral blood smear provides diagnostic clues [7]. These disorders are described below based on the inheritance pattern. Table 14.1 shows them classified by platelet size (Table 14.1). In general, platelet size >11 fl is considered macrothrombocytopenia (large platelets), platelet size between 7 and 11 fl is considered normal-sized platelets, and platelet size <7 fl is considered microthrombocytopenia (small platelets). Recently, a new classification has been proposed that more precisely categorizes inherited thrombocytopenias based on platelet diameter measurements and percentage of platelets larger or smaller than specific thresholds [8].

Beyond platelet morphology, the last two decades have witnessed an explosion in our knowledge of the numerous gene mutations linked to inherited thrombocytopenias [5, 7–13]. As of now over 20 specific genes mutations have been linked to inherited thrombocytopenias [9]. The implicated genes are also shown in Table 14.1. Some affected genes encode for hematopoietic transcription factors, which exert influence on numerous downstream target genes. Transcription factor mutations (e.g., *RUNX1*, *FLI1*, *GATA1*, *GFI1B*) can result in both inherited thrombocytopenia and platelet dysfunction.

Autosomal Dominant Thrombocytopenias

***MYH9*-Related Thrombocytopenia Syndromes** [14] Although the incidence of *MYH9*-related thrombocytopenias is unknown, they are likely the most common of the inherited thrombocytopenias and probably the best recognized. Yet, these patients are likely underdiagnosed. These disorders are caused by mutations in the *MYH9* gene that encodes non-muscle myosin heavy chain IIa and includes the May-Hegglin anomaly and other entities (Fechtner, Epstein, and Sebastian syndromes) with variably associated clinical features such as nephritis, sensorineural deafness, and cataracts. The thrombocytopenia is mild to moderate with large or giant platelets and a mild bleeding diathesis. Neutrophils have large blue cytoplasmic inclusions called Döhle-like bodies, which are non-muscle myosin heavy chain IIA in the May-Hegglin variant (Fig. 14.2).

Table 14.1 Selected inherited thrombocytopenias classified by platelet size, with implicated genetic mutation

Large/giant platelets	Normal platelets	Small platelets
(MPV >11 fl)	(MPV 7–11 fl)	(MPV <7 fl)
MYH9 thrombocytopenias[a]	*RUNX1* mutations[a]	Wiskott-Aldrich syndrome/
(e.g., May-Hegglin anomaly)		X-linked thrombocytopenia (*WAS*)[b]
Mediterranean thrombocytopenia (*GP1BA*, *GP1BB*)[a]	Amegakaryocytic thrombocytopenia with radioulnar synostosis (*HOXA11*, *MECOM*)[a]	
Paris-Trousseau thrombocytopenia (*FLI1*)[a]	Congenital amegakaryocytic thrombocytopenia (*MPL*)[b]	
Gray platelet syndrome (*NBEAL2*)[a]	Thrombocytopenia with absent radii (*RBM8A*)[b]	
Velocardiofacial/DiGeorge syndrome (22q11.2)[a]	*ANKRD26*-related thrombocytopenia	
Bernard-Soulier syndrome (*GP1BA*, *GP1BB*, *GP9*)[b]		
GATA1 mutations[c]		
TUBB1-related thrombocytopenia[a]		
ACTN1-related thrombocytopenia[a]		

The gene implicated is shown in parenthesis
MPV mean platelet volume
[a]Autosomal dominant inheritance
[b]Autosomal recessive inheritance
[c]X-linked disorder

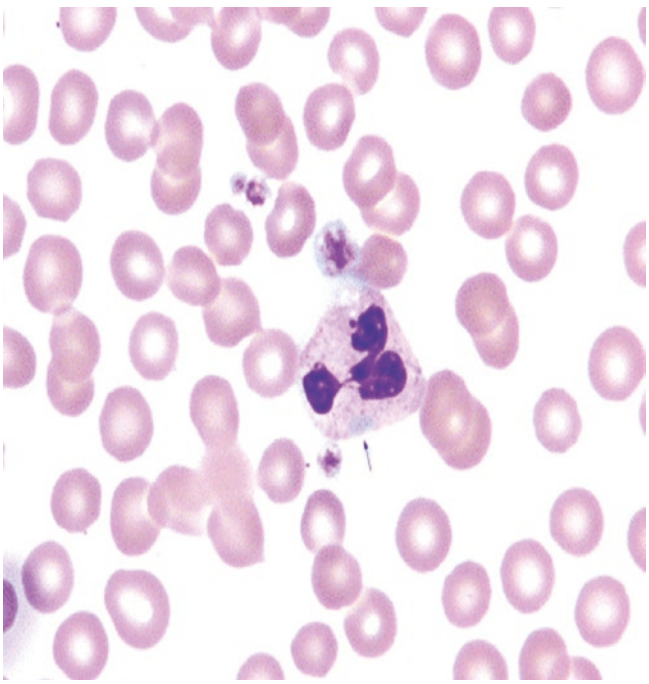

Fig. 14.2 Peripheral smear showing a giant platelet and a granulocyte inclusion called Dohle bodies in a patient with May-Hegglin syndrome. (Reprinted with permission from Lazarchick J et al. ASH Image Bank 2008; 2008: 8–00023)

Mediterranean Macrothrombocytopenia This disorder is a relatively common mild form of macrothrombocytopenia in southern Europe and is without significant bleeding. The thrombocytopenia is identified incidentally during routine blood testing. This disorder is due to a heterozygous mutation in *GP1BA*, which results in impaired expression of GPIbα, and has been considered a heterozygous form of the Bernard-Soulier syndrome [11, 15]. Recently, heterozygous variants in *GP1BB*, which encodes for GPIbβ, have also been reported, and these are associated with macrothrombocytopenia and mild bleeding manifestations [16].

***RUNX1*-Related Mutations** This is also known as familial platelet disorder with predisposition to acute myelogenous leukemia (FPD-AML) [6]. Patients have mutations or deletions in the *RUNX1* gene, a major hematopoietic transcription factor that regulates hematopoiesis and megakaryopoiesis. These patients have mild to moderate thrombocytopenia and bleeding symptoms, a familial platelet dysfunction, and a striking predisposition to acute leukemia, with an estimated 40% risk with median age of diagnosis at 33 years [17].

Paris-Trousseau Thrombocytopenia [6] Platelets are larger than normal in this disorder and are characterized by giant α-granules. It is associated with mutations involving transcription factor FLI1 located on chromosome 11q, and the bone marrow has increased immature megakaryocytes. More extensive deletion of chromosome 11q is associated with the Jacobsen syndrome, which in the vast majority of cases includes the Paris-Trousseau platelet disorder, along with syndromic features such as mental retardation, facial, cardiac, and genitourinary abnormalities.

Gray Platelet Syndrome This markedly heterogeneous [13, 18, 19] entity is characterized by reduced or absent α-granules in platelets that appear gray color on Wright-Giemsa stain. The defects are in α-granule formation and targeting of proteins to the α-granules. Some of these patients have macrothrombocytopenia. Affected patients generally have a mild to moderate bleeding diathesis.

Velocardiofacial/DiGeorge Syndrome These syndromes are caused by deletions in chromosome 22 and have only mild thrombocytopenia without significant bleeding. Additional features include cardiac abnormalities, defects in parathyroid and thymus development, cognitive impairment, and, in some, facial abnormalities.

Other Inherited Autosomal Dominant Thrombocytopenias

The application of next-generation sequencing has contributed enormously to the uncovering of novel gene mutations in inherited autosomal dominant thrombocytopenias. For example, gene mutations in *HOXA11* and *MECOM* have been shown to underlie radioulnar synostosis and amegakaryocytic thrombocytopenia (RUSAT). Such patients have congenital fusion of the proximal radius and ulna and severe thrombocytopenia due to lack of bone marrow megakaryocytes. Many patients progress to bone marrow failure and require hematopoietic progenitor cell transplant as treatment. Other gene mutations implicated in inherited autosomal dominant thrombocytopenia include *TUBB1*, *ACTN1*, and *ANKRD26*, which are all characterized by isolated thrombocytopenia [10]. *ANKRD26* mutation, like *RUNX1* mutation, appears to be associated with an elevated risk of development of hematologic myeloid malignancies [20].

Autosomal Recessive Thrombocytopenias

Congenital Amegakaryocytic Thrombocytopenia This entity is a severe thrombocytopenia (<10,000 platelet/μL) that presents in the newborn with absence of megakaryocytes in the bone marrow. Affected infants are identified within days to weeks of birth because of bleeding. Leukocyte and erythrocyte production is decreased leading to pancytopenia by the second decade. This disorder is caused by mutations in the thrombopoietin receptor *c-Mpl*.

Thrombocytopenia with Absent Radii (TAR) Affected individuals have shortened or absent forearms bilaterally due to defects in radius development with severe thrombocytopenia at birth requiring platelet transfusion. Thrombocytopenia often becomes less severe with increasing age. The genetic mutation that results in TAR has recently been found on involve the *RBM8A* gene [21].

Bernard-Soulier Syndrome This macrothrombocytopenia has marked platelet dysfunction with severe bleeding (see below). The homozygous mutation affects expression or function of the GPIb-IX-V complex, which forms the binding site on platelets for vWF.

X-Linked Disorders

Wiskott-Aldrich Syndrome (WAS) This syndrome affects males and is associated with moderate to severe thrombocytopenia with decreased platelet size, immunodeficiency, and eczema. WAS usually presents within the first year of life with bruising and bleeding and recurrent infections with eczema developing after the first year. The underlying defect is a mutation in the WAS protein (WASP) that links the cellular cytoskeleton with signal transduction pathways. The thrombocytopenia often improves after splenectomy. Hematopoietic progenitor cell transplantation is curative. Some patients with mutations in WASP exhibit a milder form of X-linked thrombocytopenia without significant eczema or immunodeficiency.

X-Linked Macrothrombocytopenia with Dyserythropoiesis Due to *GATA1* Mutation [6] This disorder has marked thrombocytopenia (10,000–40,000/μL) with bruising and severe bleeding, and a variable degree of anemia. It is caused by mutations in the hematopoietic transcription factor GATA1. The bone marrow is hypercellular with defects in the megakaryocytic and erythroid lineages. Some patients require stem cell transplant to due severely low blood counts.

Inherited Disorders of Platelet Function

General Features

Patients with inherited disorders of platelet function are characterized by mucosal bleeding (epistaxis, menorrhagia, gastrointestinal bleeding) and cutaneous bleeding. There is a wide variability in bleeding manifestations.

Inherited disorders of platelet function may arise by aberrations in multiple mechanisms [22–24]. Table 14.2 provides a classification of the well recognized entities based on the platelet function or responses that are abnormal (Fig. 14.1). Not all of the disorders listed are due to a defect in the platelets per se. Some, such as the von Willebrand disease (vWD) and afibrinogenemia, result from deficiencies of plasma proteins essential for platelet-endothelial and platelet-platelet interactions, respectively. In many patients with inherited platelet dysfunction, the underlying molecular mechanisms leading to the abnormal platelet responses remain unknown.

Disorders of Platelet Adhesion

In patients with defects in platelet-vessel wall interactions, adhesion of platelets to subendothelium is abnormal. The two disorders in this group are von Willebrand disease

Table 14.2 Inherited disorders of platelet function

1. Defects in platelet-vessel wall interaction (disorders of adhesion)
(a) von Willebrand disease (deficiency or defect in plasma vWF)
(b) Bernard-Soulier syndrome (deficiency or defect in GPIb)
2. Defects in platelet-platelet interaction (disorders of aggregation)
(a) Congenital afibrinogenemia (deficiency of plasma fibrinogen)
(b) Glanzmann thrombasthenia (deficiency or defect in GPIIb-IIIa)
3. Disorders of platelet granules, secretion and activation
(a) Disorders of granules
(i) Storage pool deficiency (δ, α, αδ)
(ii) Quebec platelet disorder
(b) Disorders of platelet activation and signal transduction mechanisms
(i) Defects in platelet agonist receptors (thromboxane A_2, ADP receptors ($P2Y_{12}$), epinephrine, platelet activating factor)
(ii) GTP-binding protein related defects (Gαq deficiency, Gαi1 deficiency, Gαs hyperfunction, CalDAG-GEF1 deficiency)
(iii) Phospholipase C-β2 deficiency and defects in phospholipase C activation, calcium mobilization, and protein phosphorylation (PKC-θ deficiency)
(c) Defects in thromboxane A_2 synthesis (deficiency of phospholipase A2, cyclooxygenase, or thromboxane synthase)
4. Disorders of platelet coagulant-protein interaction (Scott syndrome)
5. Other disorders
(a) Defects related to cytoskeletal/structural proteins Wiskott-Aldrich syndrome
(b) Abnormalities of transcription factors: mutations in *RUNX1* (familial platelet disorder with predisposition to acute myelogenous leukemia), *FLI1*, *GFI1B*, *GATA1*

(vWD) (see Chap. 12) due to a deficiency or abnormality in plasma vWF and the Bernard-Soulier syndrome (BSS) in which platelets are deficient in GPIb (and GPV and GPIX). In both disorders, platelet-vWF interaction is compromised.

Bernard-Soulier Syndrome (BSS)

BSS is a rare (estimated incidence of ~1/1,000,000) autosomal recessive platelet function disorder resulting from an abnormality in platelet GP Ib-IX-V complex, which mediates the binding of vWF to platelets [25]. GPIb exists in platelets as a complex consisting of glycoproteins Ib, IX, and V, and it is reduced in the BSS due to mutations that affect expression of GPIb or GPIX [11]. The platelet counts are moderately decreased, platelet size is markedly increased, and the bleeding time is prolonged. In platelet aggregation studies, the responses to ADP, epinephrine, and collagen are normal. Characteristically, the aggregation in PRP in response to the antibiotic ristocetin is decreased or absent, a feature shared with patients with vWD. However, unlike in vWD, plasma vWF and factor VIII are normal in BSS. The diagnosis of BSS is established by demonstrating decreased platelet surface GPIb, most commonly by flow cytometry [3, 25].

Disorders of Platelet Aggregation

Binding of fibrinogen to the GPIIb-IIIa (integrin $\alpha_{2b}\beta_3$) complex is a prerequisite for platelet aggregation, and platelet activation is required for this binding. Disorders characterized by abnormal platelet-platelet interactions (aggregation disorders) arise because of a quantitative or qualitative abnormality of the platelet membrane GPIIb-IIIa complex, which binds fibrinogen (Glanzmann thrombasthenia). Platelet aggregation is absent also in the absence of plasma fibrinogen (inherited afibrinogenemia). In general, as little as 50 μg/ml fibrinogen is adequate for normal platelet aggregation. Therefore, in most clinical situations, such as disseminated intravascular coagulation, where plasma fibrinogen is decreased, but not absent, the impact on platelet aggregation is not of major consideration. Disorders that disrupt activation of GPIIb-IIIa can also adversely affect platelet aggregation (defect in "inside-out" signaling), but are not caused by a deficiency of GPIIb-IIIa per se.

Glanzmann Thrombasthenia

Glanzmann thrombasthenia is a rare autosomal recessive disorder. The incidence is unknown but is higher in populations with higher consanguinity. It is characterized by markedly impaired platelet aggregation, a prolonged bleeding time, and severe mucocutaneous bleeding manifestations [25]. In Glanzmann thrombasthenia, there is a quantitative or qualitative defect in the GPIIb-IIIa complex arising from mutations in either the GPIIb or GP IIIa gene. Because of this defect, fibrinogen binding to platelets is decreased, and aggregation is impaired. Clot retraction, a function of the interaction of GPIIb-IIIa with the platelet cytoskeleton, is also impaired. The diagnostic hallmark of thrombasthenia is absence or marked decrease of platelet aggregation in response virtually to all agonists (except ristocetin), with absence of both the primary and the secondary wave of aggregation (Fig. 14.3). Because platelet secretion is dependent on aggregation, dense granule secretion may be decreased when platelets are activated with weak agonists (e.g., ADP, epinephrine) but normal on activation with thrombin, a potent agonist. Heterozygotes have ~50% of platelet GPIIb-IIIa complexes and have no bleeding symptoms, and the platelet aggregation responses are normal. Thus, the parents have no bleeding symptoms and have normal platelet responses on activation. The diagnosis of thrombasthenia is established by demonstrating a deficiency of the integrin receptor, generally by flow cytometry.

Inherited Afibrinogenemia

Although inherited afibrinogenemia is also characterized by an absence of platelet aggregation responses, such patients can be distinguished from those with Glanzmann thrombasthenia because the PT, APTT, and thrombin time

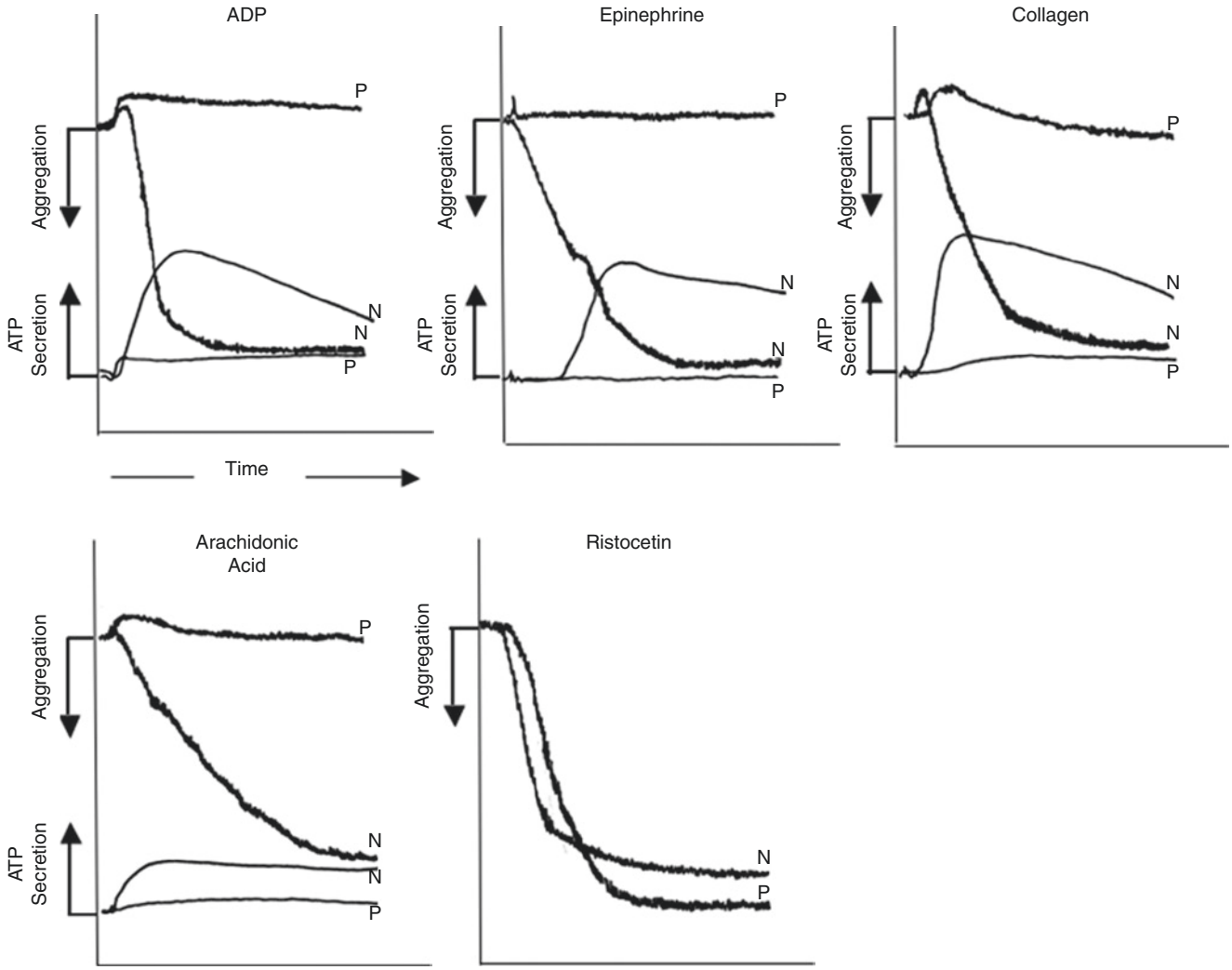

Fig. 14.3 Aggregation and ATP secretion studies in Glanzmann thrombasthenia performed using a lumi-aggregometer and platelet-rich plasma (PRP). Shown are aggregation (upper tracings in each panel) and ATP secretion (lower tracings) in the patient (P) and a healthy subject (N) in response to ADP (7.5 μM), epinephrine (7.5 μM), collagen (1 μg/ml), ristocetin (1.5 mg/ml), and arachidonic acid (1 mM). With all agonists except ristocetin, neither the primary wave nor the secondary wave of aggregation is noted in the patient, and secretion is decreased

are markedly prolonged, whereas they are normal in Glanzmann thrombasthenia.

Disorders of Platelet Granules, Secretion, and Activation

Patients lumped in this markedly heterogeneous group are generally characterized by impaired dense granule secretion and absence of the second wave of aggregation upon stimulation of PRP with ADP or epinephrine; responses to collagen, thromboxane analog (U46619), or arachidonic acid may also be decreased. Indeed, the underlying mechanisms are remarkably diverse, and this categorization is more out of convenience than an understanding of the mechanisms or genetics. Platelet function is abnormal in these patients either when the granule contents are diminished, as in storage pool deficiency (SPD) leading to decreased secretion, or when signaling mechanisms are impaired leading to decreased platelet responses, including aggregation and secretion (Table 14.2) [23, 24].

Deficiency of Granule Stores (Storage Pool Deficiency)

The term storage pool deficiency refers to patients with deficiencies in platelet contents of dense granules (δ-SPD), alpha-granules (α-SPD, gray platelet syndrome), or both types of granules (αδ-SPD).

Patients with *δ-storage pool deficiency* (δ-SPD) have a mild to moderate bleeding diathesis associated with a prolonged bleeding time [23, 24, 26]. In the platelet studies, the second wave of aggregation in response to ADP and epinephrine is usually absent or blunted, and the collagen

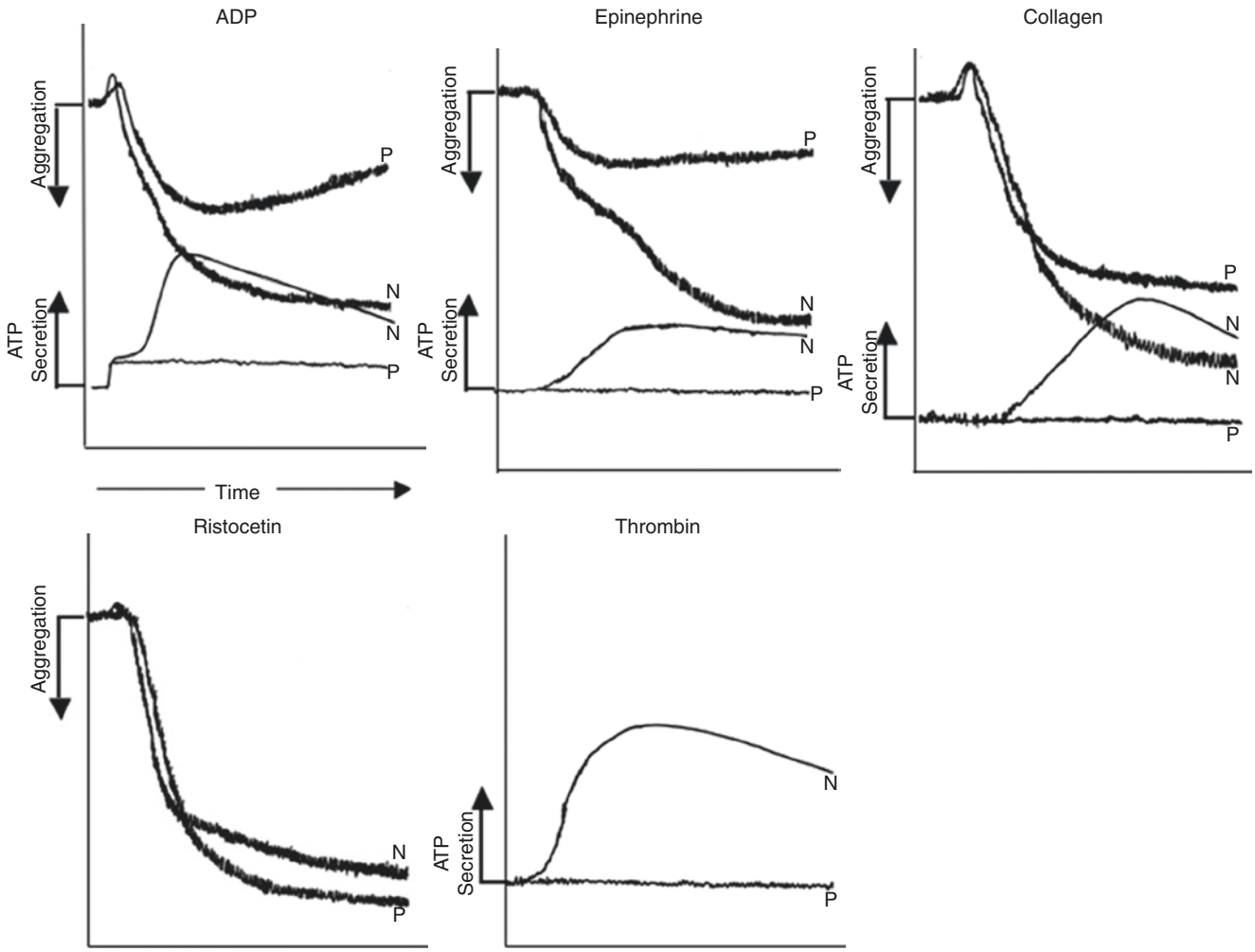

Fig. 14.4 Aggregation and ATP secretion studies in δ-storage pool deficiency. Shown are responses the patient (P) and a healthy subject (N) to ADP (7.5 μM), epinephrine (7.5 μM), collagen (1 μg/ml), ristocetin (1.5 μg/ml), and thrombin (2 U/ml). In the patient, only the primary wave of aggregation is noted with ADP and epinephrine; aggregation is blunted with collagen. Secretion is decreased with all agonists except ristocetin. With thrombin only secretion is shown because addition of thrombin induces clotting of fibrinogen and does not permit recording of aggregation

response is markedly impaired (Fig. 14.4). Normal platelets possess 3–8 dense granules (each 200–300 nm in diameter). Under the electron microscope, dense granules are decreased in SPD platelets. By direct biochemical measurements, the platelet ATP and ADP contents are decreased along with other dense granule constituents. δ-SPD may occur by itself or in association with syndromic disorders such as the Hermansky-Pudlak syndrome (HPS) (characterized by oculocutaneous albinism, nystagmus, and increased reticuloendothelial ceroid, and associated with mutations in the *HPS* and other genes), the Chediak-Higashi syndrome (defect in *LYST* or *CHS1* gene), the Wiskott-Aldrich syndrome (*WAS* gene defect), and the thrombocytopenia-absent radius (TAR) syndrome. There is a large group of HPS patients in northwest Puerto Rico. Chediak-Higashi syndrome is a rare autosomal recessive disorder characterized by SPD, oculocutaneous albinism, immune deficiency, neurological symptoms, and presence of giant cytoplasmic inclusions in different cells.

Patients with the *gray platelet syndrome (GPS*) have an isolated deficiency of platelet α-granule contents [13, 18, 19]. The platelets have a gray appearance due to the paucity of granules on the peripheral blood smears. These patients have a bleeding diathesis, mild thrombocytopenia, and a prolonged bleeding time. The platelets are deficient in α-granule proteins including platelet factor 4, β-thromboglobulin, and vWF. Platelet aggregation responses to ADP and epinephrine are normal in most patients; in some patients, aggregation responses to thrombin, collagen, and ADP are impaired.

The *Quebec platelet disorder (QPD)*, another disorder affecting the platelet α-granules, is an autosomal dominant disorder associated with delayed bleeding and abnormal proteolytic degradation of α-granule proteins (including fibrinogen, factor V, vWF, thrombospondin, multimerin,

and P-selectin) due to increased amounts of platelet urokinase type plasminogen activator (uPA). This results from duplication of a 78 kb segment that includes *PLAU*, the gene for uPA.

Defects in Platelet Activation/Signal Transduction Mechanisms

Signal transduction mechanisms encompass processes that are initiated by the interaction of agonists with specific platelet surface receptors and include responses such as G-protein activation and activation of intracellular enzymes, such as phospholipase C and phospholipase A_2 (Fig. 14.1).

Patients with receptor defects have impaired responses because of an abnormality in the platelet surface receptors for a specific agonist. Such defects have been documented for receptors for ADP ($P2Y_{12}$), thromboxane A2, collagen (GP VI or GPIa/IIa; $\alpha_2\beta_1$), and epinephrine (Fig. 14.1). Patients with the ADP receptor abnormalities have had a defect in the $P2Y_{12}$ ADP receptor, which is coupled to inhibition of adenylyl cyclase mediated by the G-protein Gαi (Fig. 14.1). G-proteins link surface receptors and intracellular effector enzymes, and *defects in G-protein activation* have impaired signal transduction. Patients with platelet defects in G-proteins Gαq, Gαi1 and Gαs, and calcium and diacyglycerol-regulated guanine exchange factor I (CalDAG-GEFI) have been described. Patients have been described with impaired signal transduction due to *defects in phospholipase C activation*, *calcium mobilization*, *pleckstrin phosphorylation*, and other mechanisms (Fig. 14.1). Defects in phospholipase C-β2 and protein kinase C-θ have been documented.

A major platelet response to activation is liberation of arachidonic acid from phospholipids and its subsequent oxygenation to thromboxane A2. Patients have been described with impaired thromboxane synthesis due to *inherited deficiencies of cyclooxygenase* and *thromboxane synthase* and *phospholipase* A_2. In addition to the above, several other inherited abnormalities have been recently documented in different genes and proteins that regulate structure, number, and function of platelets. These are reviewed elsewhere [9, 23].

Disorders of Platelet Procoagulant Activities

Platelets provide the surface on which several specific key enzymatic reactions of blood coagulation occur (Fig. 14.1), and this role of platelets is called platelet procoagulant activity. Platelet activation induces a redistribution of phospholipids with expression of phosphatidylserine on the outer surface. This phospholipid translocation is essential to the expression of platelet procoagulant activities. A few patients have been described in whom this platelet contribution to blood coagulation is impaired, and this is referred to as the *Scott syndrome* [23, 24, 27]. In these patients the bleeding time and platelet aggregation responses have been normal.

Other Abnormalities

It is being increasingly recognized that in some patients with inherited platelets defects, the gene defect is in a hematopoietic transcription factor that regulates gene expression in megakaryocytes and platelets [6]. Mutations in transcription factors RUNX1, FLI1, GATA1, GFI1B, ETV6, EVI1, and HOXA11 have all been associated with thrombocytopenia, with some also associated with impaired platelet function [6].

Management of Inherited Platelet Function Defects

Because of the wide variation in the severity of bleeding manifestations, management of these patients needs to be individualized. Platelet transfusions are indicated in the management of clinically relevant bleeding and in preparation for surgical procedures. In addition to the usual risks associated with transfusions, alloimmunization to specific missing platelet glycoproteins may occur in patients with Glanzmann thrombasthenia and the Bernard-Soulier syndrome. These patients may therefore develop antibodies against glycoproteins that compromise the efficacy of subsequent platelet transfusions. An alternative to platelet transfusions is intravenous administration of DDAVP, which induces the release of vWF from endothelial cells and shortens the bleeding time in some patients with platelet function defects. DDAVP is not currently FDA approved for management of patients with inherited platelet defects. Infusion of recombinant factor VIIa has been used in the management of bleeding events in patients with platelet disorders, particularly those with Glanzmann thrombasthenia and alloantibodies against platelet glycoproteins.

Summary

Platelets play a vital role in hemostasis. Patients with inherited platelet disorders are at increased bleeding risk at mucocutaneous sites and with hemostatic challenges, such as surgery or trauma. Such patients are not uncommon, though they are underdiagnosed. The two categories of inherited platelet disorders are those associated with decreased platelet number (thrombocytopenia) and those characterized by impaired platelet function (Fig. 14.5). Some patients

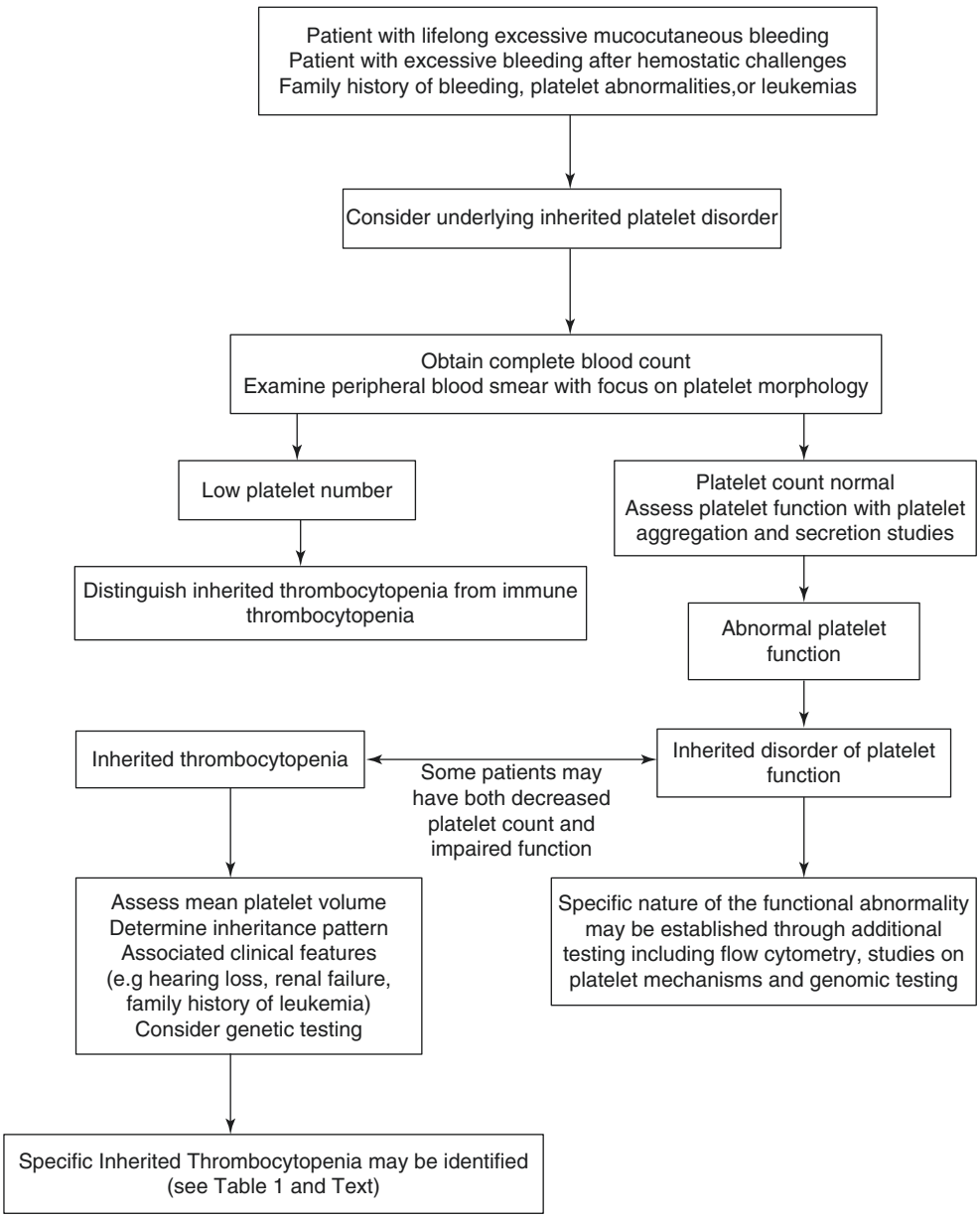

Fig. 14.5 Algorithm on recognition and identification of an inherited platelet disorder

may have a combination of thrombocytopenia and platelet dysfunction on an inherited basis. Inherited thrombocytopenias can be categorized based on mean platelet volume and/or inheritance patterns; associated clinical features can help distinguish between the different inherited thrombocytopenias. They need to be distinguished from patients with immune thrombocytopenic purpura. Inherited disorders of platelet function may arise from several molecular mechanisms, and in many such patients, the aberrant platelet mechanisms are unknown. Management of patients with inherited platelet disorders needs to be individualized based on the severity of the bleeding symptoms; the options include platelet transfusions, DDAVP, and, in select cases, recombinant factor VIIa.

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Acquired Thrombocytopenia

15

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Introduction

Thrombocytopenia (combination of medical terms "thrombocyte" [*platelet*] and "penia" [*deficiency*], based upon Greek "thrombos" [*clot*], "kytos" [*container*, with modern meaning of *cell*], and "penia" [*poverty*]) indicates reduced platelet count numbers. Depending on its cause, thrombocytopenia indicates increased risk of bleeding, thrombosis, and/or mortality. Thus, when faced with a thrombocytopenic patient, the clinician's key question is: *what is the probable cause of the patient's thrombocytopenia?* The answer will point to the appropriate prognostic and therapeutic considerations.

Definition

The normal platelet count range is approximately 150,000–400,000/μL (150–400 × 10^9/L); thus, "thrombocytopenia" usually indicates a platelet count <150,000/μL. Conceptually, thrombocytopenia infers any pathological reduction in platelet numbers, and thus a large decrease in the platelet count that does not necessarily fall below 150,000/μL can also indicate thrombocytopenia, e.g., a >50% platelet count fall from 400,000 to 175,000/μL during the second postoperative week as a sign of heparin-induced thrombocytopenia (HIT) [1]. Thrombocytopenia can be classified as mild (100,000–150,000/μL), moderate (50,000–100,000/μL), severe (<50,000/μL), or very severe (<20,000/μL).

Clinical Features of Thrombocytopenia

A. *Hemorrhage* is a potential complication of thrombocytopenia (Table 15.1).
B. *Sites of bleeding:*
   1. *Cutaneous* (various types of purpura, e.g., petechiae [pinpoint hemorrhages located in regions of increased hydrostatic pressure, e.g., dorsa of feet in ambulatory patients, lower back in bedridden patients, upper chest/face in ventilated patients] or ecchymoses [purpura >2 mm in diameter], often associated with trauma, e.g., venipuncture sites)
   2. *Mucosal* (epistaxis, menometrorrhagia [heavy, irregular menstrual periods], hemorrhagic bullae in mouth [blood blisters], gastrointestinal [GI] or genitourinary [GU] bleeding)
   3. *Central nervous system (CNS)*

Table 15.1 Clinical manifestations of thrombocytopenia

Clinical context	Clinical manifestations
Bleeding (increased risk seen with many causes of thrombocytopenia)	
Platelet count, <75,000–100,000/μL	Increased bleeding with surgery and trauma
Platelet count, <20,000/μL	Increased risk of spontaneous mucocutaneous hemorrhage
Platelet count, <10,000/μL	Increased risk of spontaneous intracranial hemorrhage
Thrombosis (increased risk depends on particular cause of thrombocytopenia)	
Heparin-induced thrombocytopenia	Thrombosis, especially large veins and arteries
Disseminated intravascular coagulation	Thrombosis, especially microvascular
Thrombotic microangiopathy (TTP, HUS)	Thrombosis, especially small arteries/arterioles (CNS, renal)

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C. *Thrombosis* is a feature of certain thrombocytopenic disorders (Table 15.1).
   1. *Large vein and/or artery thrombosis* is typical of HIT [2] and cancer-associated disseminated intravascular coagulation (DIC) [3].
   2. *Microvascular thrombosis* can complicate thrombocytopenia associated with DIC; sometimes, acral (distal extremity) ischemic necrosis occurs despite palpable limb pulses [4].
   3. *Small artery/arteriolar thrombosis* is characteristic of thrombotic thrombocytopenic purpura (TTP) and hemolytic-uremic syndrome (HUS) [5].

D. *Increased mortality* is associated with thrombocytopenia in critically ill patients with multi-organ failure.

Classification of Thrombocytopenia

There are five general mechanisms of thrombocytopenia (Table 15.2), plus pseudothrombocytopenia:

A. *Decreased platelet production* (marrow disorders)
B. *Hemodilution* (e.g., postoperative)
C. *Sequestration* (hypersplenism)
D. *Increased platelet consumption* (thrombin- and von Willebrand factor [VWF]-mediated)
E. *Increased platelet destruction* (immune-mediated)
F. *Pseudothrombocytopenia* (spurious thrombocytopenia)

Decreased Platelet Production

A. *Pancytopenia*

Many bone marrow disorders feature reduction in circulating red cells, white cells, and platelets ("pancytopenia") (Table 15.3). Bone marrow megakaryocytes are usually decreased in number, either in the setting of marrow aplasia/hypoplasia or because of replacement by fibrosis, tumor, or hypercellular (megaloblastic) marrow.

1. *Primary bone marrow disorders (congenital/hereditary)* (see Chaps. 13 and 18):
   (a) Fanconi's anemia
   (b) Miscellaneous hereditary aplastic anemia
   (c) Congenital (intrauterine) infections
2. *Primary bone marrow disorders (acquired)* (see Chaps. 24, 25, 26, and 27):
   (a) Acquired aplastic anemia
   (b) Leukemia
   (c) Myelodysplasia
3. *Bone marrow infiltration*:
   (a) Metastatic cancer to the bone marrow, most often breast and prostate cancer, causes "myelophthisis," manifesting as leukoerythroblastosis (left shift in circulating granulocytes—metamyelocytes, myelocytes, promyelocytes, and rarely myeloblasts—and nucleated red blood cells) and "tear drop" red cells (dacrocytes) [6]; sometimes, the clinical picture resembles TTP [7].

Table 15.2 Classification of thrombocytopenia

Classification	Picture	Mechanisms
Decreased platelet production	Pancytopenia or isolated thrombocytopenia[a]	Injured or abnormal stem cells result in reduction in all three cell lines
Hemodilution	Bicytopenia or pancytopenia	Administration of fluids, especially during surgery, associated with abrupt decrease in concentration of platelets and red cells; white cell count may remain normal or increase due to acute inflammation
Sequestration	Pancytopenia	Hypersplenism (trapping of all three cell lines in enlarged spleen)
Increased platelet consumption	Isolated thrombocytopenia (DIC) or thrombotic microangiopathy (TTP, HUS)	Increased thrombin generation explains thrombocytopenia in DIC; increased VWF-platelet-subendothelial interactions are associated with thrombotic microangiopathy in TTP and HUS
Increased platelet destruction	Isolated thrombocytopenia	Antibody-mediated platelet clearance by mononuclear-phagocytic (reticuloendothelial) system (auto-, allo-, and drug-dependent antibodies); exception: platelet-activating antibodies characterize HIT
Pseudothrombocytopenia	Platelet clumps or platelet rosetting on neutrophils	Spurious thrombocytopenia that results when electronic particle counter fails to identify platelets

[a]Certain disorders of decreased platelet production result in isolated thrombocytopenia, rather than pancytopenia: e.g., MYH9-associated macrothrombocytopenia, β1-tubulin macrothrombocytopenia, alcohol-induced thrombocytopenia, acquired amegakaryocytic thrombocytopenia, and some patients with myelodysplasia

Table 15.3 Thrombocytopenia caused by decreased platelet production

Congenital/hereditary	Acquired
Pancytopenia	
Primary bone marrow disorders Fanconi anemia Miscellaneous aplastic anemias Dyskeratosis congenita Shwachman-Diamond syndrome Congenital intrauterine infection Rubella Cytomegalovirus Herpes virus Echovirus Toxoplasmosis Syphilis	Primary bone marrow disorders: Acquired aplastic anemia, leukemia, myelodysplasia Bone marrow infiltration: Metastatic cancer Myelofibrosis Infectious diseases (e.g., tuberculosis) Bone marrow injury: Drugs (e.g., chemotherapy, chloramphenicol) Chemicals (e.g., benzene) Radiation Megaloblastic anemia (B_{12} or folate deficiency) Copper deficiency
Isolated thrombocytopenia	
Thrombocytopenia-absent radius (TAR) syndrome Miscellaneous syndromic hypomegakaryocytic thrombocytopenia Congenital amegakaryocytic thrombocytopenia Wiskott-Aldrich syndrome (WAS) and variant WAS MYH9-associated thrombocytopenia[a] β1-tubulin macrothrombocytopenia[a]	Alcohol-induced thrombocytopenia Iron depletion or repletion Acquired amegakaryocytic thrombocytopenia

[a]Hereditary thrombocytopenia may not be recognized until adulthood, and thus mimic an "acquired" thrombocytopenia, if previous platelet count values from childhood and infancy are not available

(b) Myelofibrosis with myeloid metaplasia also produces myelophthisis due to fibrotic infiltration (see Chap. 18).

(c) Infectious diseases sometimes cause marrow infiltration by microbes, e.g., disseminated tuberculosis or histoplasmosis in AIDS.

4. *Bone marrow injury.* Rapidly dividing marrow progenitor cells are susceptible to a variety of injuries:

(a) Drugs. Antineoplastic agents produce predictable, transient pancytopenia; a few drugs (e.g., chloramphenicol, penicillamine, gold, carbamazepine) rarely cause idiosyncratic (immune-mediated) aplastic anemia.

(b) Chemicals such as benzene can cause bone marrow injury.

(c) Radiation causes predictable pancytopenia and, in large amounts, produces fatal marrow failure.

5. *Nutritional disorders:*

(a) Megaloblastic anemia due to vitamin B_{12} or folate deficiency can produce pancytopenia; marked red cell macrocytosis is a clue (See Chap. 6).

(b) Copper deficiency complicating total parenteral nutrition or after bowel surgery is a rare cause of pancytopenia.

B. *Isolated Thrombocytopenia*

Certain megakaryocytic disorders produce isolated thrombocytopenia (Table 15.3). Bone marrow aspirate/biopsy can reveal a "hypomegakaryocytic" picture (reduced/absent megakaryocytes), whereas normal megakaryocyte numbers suggest ineffective thrombopoiesis.

1. *Hypomegakaryocytic*

(a) Thrombocytopenia-absent radius (TAR) syndrome features bilateral absent radii and hypomegakaryocytic thrombocytopenia (also see Chap. 14). Thrombocytopenia is usually most severe during the first year of life, improving subsequently. Occasionally thrombocytopenia begins in adulthood.

(b) Miscellaneous syndromic hypomegakaryocytic thrombocytopenia includes trisomy 18, trisomy 13, and radioulnar synostosis (proximal fusion of ulna and radius), among others.

(c) Congenital amegakaryocytic thrombocytopenia without skeletal or other congenital abnormalities is a rare autosomal recessive disorder involving mutations in c-mpl (thrombopoietin receptor) [8] (also see Chap. 14).

2. *Microthrombocytopenia*

(a) Wiskott-Aldrich syndrome (WAS) is an X-linked immunodeficiency disorder characterized by eczema, recurrent infections, increased risk of malignancy, and thrombocytopenia with small platelets caused by mutations in WAS protein (WASp). Stem cell transplantation is curative [9] (also see Chap. 14).

(b) X-linked thrombocytopenia (variant WAS) is a less severe version of WAS also due to mutations in the WASp gene (also see Chap. 14).

3. *MYH9-associated macrothrombocytopenia* is autosomal dominant and caused by mutations in non-muscle myosin heavy-chain-9 [10] (see Chap. 14). Platelet size is greatly increased, causing falsely low platelet

counts by electronic particle counters. Patients are often misdiagnosed as having immune (idiopathic) thrombocytopenic purpura (ITP). As the thrombocytopenia may not be recognized until adulthood, the patient is often wrongly considered to have an acquired thrombocytopenia. Petechiae are uncommon, whereas ecchymoses and menorrhagia often occur. Depending on the specific mutation, a variety of overlapping syndromes are recognized.

4. *β1-tubulin macrothrombocytopenia* is autosomal dominant and caused by mutations in β1-tubulin [10]. Bleeding symptoms are often relatively mild.
5. *Hereditary thrombocytopathic disorders* can feature thrombocytopenia, for example, gray platelet syndrome (absence of platelet α-granules) and Bernard-Soulier syndrome (absence of the GP Ib/IX/V complex) (see Chap. 14) [10].
6. *Alcohol-induced thrombocytopenia* indicates acute, self-limited thrombocytopenia due to heavy drinking. The platelet count begins to rise 2–3 days after cessation of alcohol consumption, with "rebound" thrombocytosis. Bone marrow aspirate reveals reduced megakaryocyte numbers and sometimes vacuolation of normoblasts and promyelocytes.
7. *Iron depletion or repletion* can cause mild-to-moderate thrombocytopenia, either evident at presentation of severe iron deficiency anemia or as a transient platelet count decline seen approximately 1 week after beginning iron replacement therapy [11]. More commonly, iron deficiency is associated with thrombocytosis—increased platelet number that corrects upon iron replacement.
8. *Acquired amegakaryocytic thrombocytopenia* features isolated thrombocytopenia with marked reduction or absence of megakaryocytes. Autoimmune mechanisms are implicated in some patients, and therapy with antithymocyte globulin, high-dose corticosteroids, danazol, and/or cyclosporine may be of benefit [12].

Hemodilution

A. *Administration of fluid (crystalloid, colloid) and/or blood products*, usually in surgery/trauma settings, is a common explanation for thrombocytopenia. The platelet count nadir (lowest value) is reached 1–4 days (median, day 2) following surgery (Fig. 15.1). Rebound thrombocytosis—reaching levels ~2 to 3 times the baseline (preoperative) platelet count—occurs at approximately day 14 [1].

B. *Gestational thrombocytopenia.* The platelet count decreases by ~10 to 20% during normal pregnancy; given the ~30 to 50% increase in plasma volume during pregnancy, physiological hemodilution contributes to mild gestational thrombocytopenia in women with low-normal pre-pregnancy platelet counts [13].

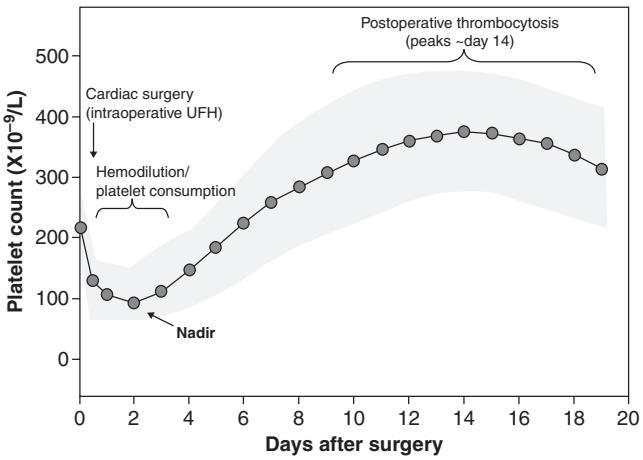

Fig. 15.1 Timeline of postoperative thrombocytopenia and thrombocytosis. Intra-/postoperative administration of crystalloid, colloid, and blood products results in *dilutional thrombocytopenia*, with the platelet count nadir reached between postoperative days 1–4, inclusive (median, day 2). Subsequently, postoperative thrombocytosis occurs, with platelet counts reaching levels approximately 2–3 times the preoperative baseline, and peaking usually at approximately day 14, with return to baseline over the subsequent 1–2 weeks. The closed circles and shaded area indicate mean ± 1 SD

Sequestration (Hypersplenism)

A. *Hypersplenism*

Hypersplenism—the reduction in one or more peripheral blood counts due to sequestration within an enlarged spleen—is a common cause of chronic thrombocytopenia. Hypersplenism arises with inflamed or congested spleen, but not with infiltrative disease such as that seen with metastatic cancer.

1. *Pathophysiology.* Usually, one-third of total body platelets reside within the spleen, even though the spleen receives only 5% of cardiac output. This discrepancy results because the splenic transit time of a platelet averages 20 min, as opposed to ~1 min for a platelet to return to the heart after passing through other organs. In hypersplenism, the proportion of platelets within the spleen can far exceed one-third (as high as ~90%).
2. *Clinical features* include splenomegaly and usually mild-to-moderate pancytopenia. The normal spleen is ~11 cm in length, but the spleen usually needs to be enlarged by at least 2 or 3 cm to be palpable, and thus diagnosis is most reliably made through imaging studies (e.g., ultrasound). Often, the degree of anemia is not as marked as neutropenia and thrombocytopenia. Especially severe thrombocytopenia can indicate advanced cirrhosis (with reduced thrombopoietin levels) and/or hyperfibrinolysis.

3. *Etiology* of hypersplenism in North America usually reflects primary hepatic disease (alcoholic cirrhosis, chronic viral hepatitis, etc.), whereas in some parts of the world, other factors are common (e.g., schistosomiasis). Causes of hypersplenism include congestive splenomegaly (Banti's syndrome), splenic vein thrombosis, Felty's syndrome associated with rheumatoid arthritis, myeloproliferative or myelodysplastic disorders, malaria, chronic myelogenous leukemia, and kala azar (leishmaniasis). Severe liver disease can be associated with a deficiency of thrombopoietin (which is produced by the liver), which may be an additional contributing factor for thrombocytopenia in addition to hypersplenism [14].
4. *Treatment* is usually not required, as thrombocytopenia rarely reaches clinically important levels, and splenectomy is not without risk.

B. *Hypothermia* in the setting of surgery, exposure, or hypothalamic dysfunction is reported to cause thrombocytopenia in both infants and adults; platelet counts correct upon rewarming [15]. Transient hepatosplenic sequestration was identified by platelet kinetic studies in animals.

Increased Platelet Consumption

Shortened platelet life span arising from mechanisms accelerated beyond that seen in normal physiology can be described as "consumptive" disorders. Pathologically increased thrombin generation—often called "disseminated intravascular coagulation (DIC)" or "consumptive coagulopathy"—usually produces thrombocytopenia, often severe. Decreased platelet survival (with or without overt DIC) due to macrophage-mediated clearance is common in sepsis. VWF-mediated platelet clearance seen in thrombotic microangiopathy (TMA) will be classified as a platelet consumption disorder.

A. *Disseminated Intravascular Coagulation*
The central concept of DIC is systematic activation of coagulation (dysregulated thrombin generation) [16]. DIC is not a single entity, but rather comprises several heterogeneous syndromes with many different triggers and potentiators. In order to diagnose DIC, the patient must have an underlying disorder associated with DIC, as well as laboratory evidence of generation of fibrin(ogen) degradation products and accelerated—usually decompensated—consumption of one or more elements of the hemostatic mechanism (thrombocytopenia, hypofibrinogenemia, elevated international normalized ratio [INR], or activated partial thromboplastin time [APTT]).
1. *Acute DIC (also see* Chap. 13) has numerous causes, including:
   (a) Trauma, burns, and shock (circulatory, septic) are a common cause of DIC in hospitalized patients.
   (b) Infection, most notably meningococcemia, where acral and non-acral tissue necrosis results from acquired protein C depletion and associated microvascular thrombosis (purpura fulminans) [4].
   (c) Obstetrical complications such as placental abruption, amniotic fluid embolism, preeclampsia/eclampsia, puerperal sepsis, and saline-induced abortion.
   (d) Malignancy, e.g., promyelocytic leukemia (see also chronic DIC).
   (e) Allergic/anaphylactic reactions.
   (f) Heparin-induced thrombocytopenia (HIT) features increased in vivo thrombin generation, but in 10–15% of cases, overt (decompensated) DIC with low fibrinogen and/or elevated international normalized ratio (INR) occurs.
   (g) Severe hemolysis, e.g., due to incompatible blood transfusion.

   Recently, acute ischemic hepatitis ("shock liver") has been identified as a key risk factor explaining acral (distal extremity) necrosis in patients with acute DIC, usually in the setting of septic or cardiogenic shock. Severe depletion in two natural anticoagulants—protein C and antithrombin—occurs due to their decreased production (secondary to transiently impaired hepatic synthetic function) and increased consumption (secondary to DIC) [4].
2. *Chronic DIC* (see also Chap. 13) has numerous triggers, including:
   (a) Malignancy, especially metastatic adenocarcinoma: patients often develop venous and/or arterial thrombosis and thrombocytopenia that improves with unfractionated heparin (UFH) or low-molecular-weight heparin (LMWH) anticoagulation; warfarin can produce microthrombosis due to protein C depletion (warfarin-associated venous limb gangrene) [3, 4].
   (b) Obstetrical complications, particularly the dead fetus syndrome, can cause chronic DIC and bleeding when delivery eventually occurs.
   (c) Kasabach-Merritt syndrome denotes the combination of hemangioma (usually in the skin) and consumptive thrombocytopenia/coagulopathy, often presenting at birth or infancy. Although the blood picture is evocative of DIC, the disorder represents *localized* (not disseminated) consumptive coagulopathy.
   (d) Abdominal aortic aneurysm often with ulcerated plaque and adherent thrombus can manifest as thrombocytopenia and consumptive coagulopathy (localized consumptive coagulopathy).

B. *Sepsis from Bacteremia, Fungemia, or Parasitemia (±DIC)*
Often, microbial invasion of the bloodstream is complicated by consumptive thrombocytopenia, with or without concomitant DIC. It is for this reason why blood cultures should be performed in patients (usually hospitalized) with unexplained acute illness and thrombocytopenia. When DIC is present, thrombin likely contributes to the thrombocytopenia (thrombin is a potent platelet agonist). However, in the absence of DIC, increased macrophage-mediated platelet clearance and, perhaps, decreased platelet production occur. Thrombocytopenia that occurs after 2–3 weeks of hospitalization, especially in a patient who has received antibiotics, suggests fungemia (e.g., candidemia). Worldwide, malaria is the most common parasite causing thrombocytopenia.

C. *Cardiovascular Disorders*
Increased platelet consumption has been linked to cardiovascular disorders.

1. *Cardiopulmonary bypass (CPB).* The use of CPB ("heart-lung machine") is associated with an average of 50% (range, 30–70%) decline in platelet count, although mostly due to hemodilution, with a small component of platelet consumption within the device itself. Bleeding results from thrombocytopenia, platelet dysfunction, and surgical factors.
2. *Catheters and prostheses.* Platelet life span is somewhat reduced in patients with prosthetic heart valves, pulmonary artery and other intra-arterial catheters, and Dacron vascular grafts, but thrombocytopenia is uncommon due to compensatory increase in platelet production.
3. *Congenital heart disease.* Decreased platelet life span and thrombocytopenia are seen in cyanotic congenital heart disease, particularly with marked hypoxemia and polycythemia.
4. *Valvular heart disease* can evince mild thrombocytopenia due to increased platelet consumption. (However, GI bleeding from angiodysplasia associated with aortic stenosis—Heyde's syndrome—results not from thrombocytopenia but rather from acquired deficiency of the largest VWF multimers due to increased VWF proteolysis/clearance at high shear caused by aortic stenosis; aortic valve replacement rapidly and permanently corrects the VWF multimer profile and thereby cures the GI bleeding [17].) (See Chap. 12.)
5. *Primary pulmonary hypertension* can be complicated by increased platelet consumption due to unknown mechanisms.
6. *Pulmonary embolism* occasionally features moderate or even severe thrombocytopenia, due either to DIC [18] or because of associated HIT [1].

D. *Thrombotic Microangiopathy*
Thrombotic microangiopathy (TMA), also known as microangiopathic hemolytic anemia (MHA), is characterized by schistocytic hemolysis and thrombocytopenia (schistocytes are red cell fragments, including triangular forms and "helmet" cells) (Atlas Figs. 17 and 18) [19, 20].

1. *Thrombotic thrombocytopenic purpura (TTP)* is characterized by TMA and organ dysfunction due to platelet-VWF aggregates within arterioles. Affected organs include the brain (confusion, dysarthria, stroke), kidneys (oliguric renal failure), heart (dysrhythmias, infarction, cardiac arrest), etc. Thrombocytopenia is often very severe (platelet count <20,000/μL).
   (a) Idiopathic TTP is most common and often accompanied by acquired deficiency of the VWF-cleaving protease, ADAMTS13 (a disintegrin and metalloprotease with thrombospondin-like motifs-13). In about 10% of patients, it can also be congenital (Upshaw-Schulman syndrome) due to ADAMTS13 deletions, mutations, and missense defects. Risk factors for TTP include female sex and African-American race; most patients are middle-aged. Usually, there is little activation of coagulation (no/minimal evidence for DIC). Serum lactate dehydrogenase levels (marker of intravascular hemolysis) and platelet counts are useful parameters to judge disease activity and response to treatment. Primary therapy includes plasma exchange and high-dose corticosteroids. Rituximab (off-label indication) appears effective for refractory/recurrent TTP if the condition is acquired and anti-ADAMTS13 autoantibodies are present.
   (b) Autoimmune disorders, particularly systemic lupus erythematosus (SLE) can be complicated by TTP or a TTP-mimicking disorder [21]. The autoimmune disorder can present several years before, contemporaneous with, or after the episode(s) of TTP.
   (c) Pregnancy/postpartum TTP. Pregnancy is a risk factor for TTP [22].
   (d) Post-surgery/post-pancreatitis TTP suggests that acute inflammation rarely can trigger TTP, as affected patients develop TMA 2–7 days later.
   (e) Drug-induced and hematopoietic stem cell transplantation-associated TTP. Quinine (most often), chemotherapy (e.g., gemcitabine, pentostatin, mitomycin C), and (perhaps) clopidogrel and ticlopidine can cause TMA [23]. Distinguishing the role of drugs versus effects of transplantation can be difficult when TMA occurs post-marrow

transplantation. Whereas quinine can trigger TTP abruptly, other drugs (gemcitabine, mitomycin) require significant drug accumulation. Transplantation-associated TMA is not caused by ADAMTS13 deficiency, and plasma exchange is usually ineffective [24].

2. *TTP-mimicking disorders* include:
   (a) Paravalvular leak ("macroangiopathic" hemolysis) is suggested by schistocytic hemolysis without thrombocytopenia beginning weeks or months after cardiac valve repair/replacement.
   (b) HIV infection can produce a TMA picture.
   (c) Malignant hypertension: Approximately one-quarter of patients with malignant hypertension have associated TMA.
   (d) Vasculitic disorders, e.g., systemic sclerosis and Wegener's granulomatosis, can be associated with TTP.
   (e) HELLP syndrome (hemolytic anemia, elevated liver enzymes, low platelets) is an obstetric TMA disorder associated with preeclampsia with increased fetal/maternal morbidity and mortality.
   (f) Metastatic malignancy (often involving the bone marrow) [7].
3. *Hemolytic-uremic syndrome (HUS)* is characterized by TMA plus prominent renal dysfunction. Despite clinical overlap with TTP, the lack of reduced ADAMTS13 levels and the unique bacterial trigger indicate a distinct etiology and pathogenesis.
   (a) Typical HUS indicates preceding bloody diarrhea (D+ HUS) triggered by certain bacteria, most often verocytotoxin-producing *Escherichia coli* O157:H7 and O104:H4 [25] and *Shigella*. Most patients are at extremes of age (children, elderly). Compared with TTP, oliguric renal failure is more prominent, and nonrenal involvement is less common. The bacterial infection arises from diet (e.g., unwashed/raw spinach, contaminated/undercooked hamburger meat, bean sprouts).
   (b) Atypical HUS (aHUS) or nonenteropathic HUS (D– HUS) indicates HUS associated with complement system dysregulation, especially in the constitutively active alternative pathway (e.g., mutations in complement factor H, factor I, membrane complement protein (MCP), thrombomodulin, C3, factor B) (Fig. 15.2); approximately 50% of patients with aHUS have a complement factor mutation [26]. Compared with typical HUS, there is no prodromal GI illness, and patients are at greater risk of permanent renal failure. These patients primarily present with a microangiopathic hemolytic anemia and renal failure and have less prominent thrombocytopenia. Only approximately half of the patients with these genetic abnormalities develop aHUS (50% penetrance), indicating that other factors (e.g., infection, pregnancy) are required to initiate the microangiopathic process. Eculizumab is a monoclonal antibody directed against complement component C5. It is an effective, FDA-approved therapy that can improve hematological parameters and renal function in patients with aHUS [27].

Increased Platelet Destruction

Increased platelet destruction indicates reduced platelet life span caused by autoantibodies, alloantibodies, or drug-dependent antibodies, which target platelet surface glycoproteins or other structures.

A. *Immune Thrombocytopenia (ITP)*
   The classic 1951 report of Harrington and coworkers [28] describing abrupt, severe thrombocytopenia following infusion of plasma from ITP patients into normal subjects proved a humoral factor—subsequently identified as platelet-reactive IgG—as the cause of ITP. The antibodies usually bind to platelet surface glycoproteins IIb/IIIa and/or GPIb/IX through their Fab "arms," producing reticuloendothelial clearance when antibody Fc bind to macrophages Fc receptors. Thrombopoietic agents in ITP increases impaired platelet production which is recognized as an additional pathogenetic factor in the disorder. The fundamental trigger for ITP is unknown. The clinical picture differs between children and adults.
   1. *ITP of childhood* usually follows a viral illness and is characterized by severe, but usually transient, thrombocytopenia. Corticosteroids and/or high-dose IVIg are given for severely symptomatic children, but many children can be managed with careful observation since the platelet count often recovers on its own. Chronic ITP in childhood (duration >12 months) resembles ITP in adults. But unlike adults, splenectomy is usually avoided in children (greater risk of post-splenectomy sepsis).
   2. *ITP in adults* can be classified as newly diagnosed (duration, 0–3 months), persistent (3–12 months), or chronic (>12 months). ITP in adults tends to become chronic.
   3. *Chronic ITP in adults* usually occurs in middle-aged or elderly patients, with female predominance. The bone marrow is typically normal, and splenomegaly is not found. Most ITP is "primary," but the presence of

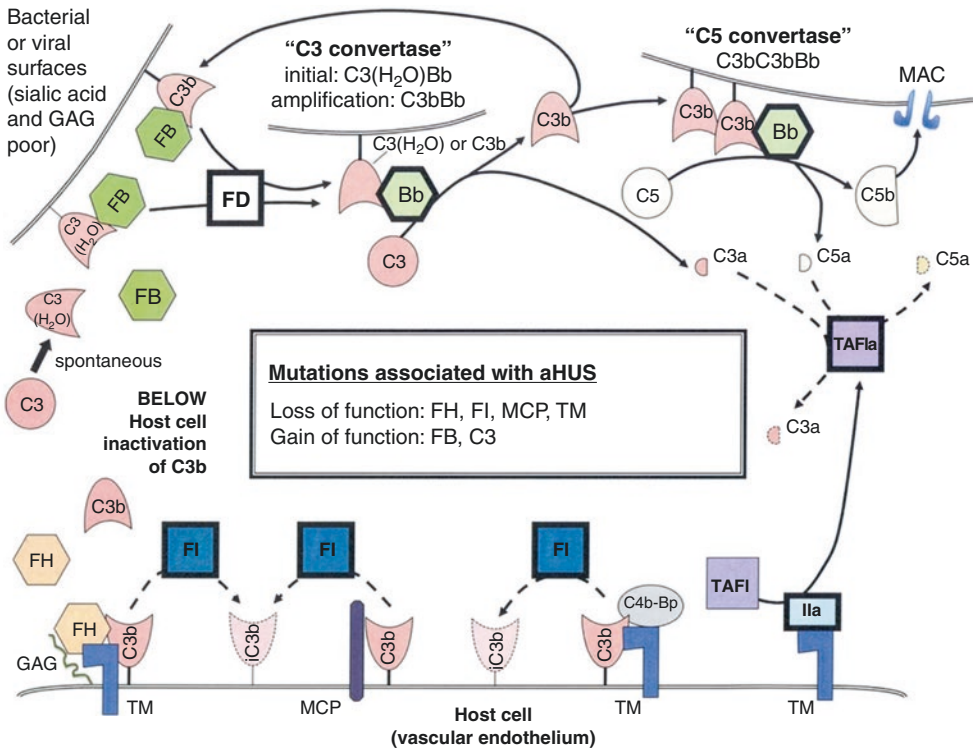

Fig. 15.2 The alternative complement pathway: implications for pathogenesis of aHUS. The central molecule of the complement cascade, C3 (see far left of figure), undergoes a continuous low level of spontaneous hydrolysis, yielding $C3(H_2O)$, which is "C3b-like." When $C3(H_2O)$ binds to an abnormal *activating* cell surface (i.e., lacking sialic acid and glycosaminoglycan [GAG]), such as a bacterium or virus (see top left of figure), the $C3(H_2O)$ binds factor B (FB), forming a complex which is cleaved by factor D (FD) to form $C3(H_2O)Bb$, i.e., the "initial" C3 convertase of the alternative pathway). This initial C3 convertase cleaves C3 into C3b (the key effector molecule of the complement system) and C3a (anaphylatoxin). C3b also binds to FB, and the complex is cleaved by FD to form C3bBb, i.e., the "amplification" C3 convertase of the alternative pathway. A positive feedback loop is created, as the initial constitutive generation of low levels of $C3(H_2O)$ leads to formation of much greater amounts of C3b via this process. If sufficient C3b is generated, some binds to C3bBb, forming "C5 convertase" (C3bC3bBb), which converts C5 to C5a (anaphylatoxin) and C5b; ultimately, if formed in sufficient quantities, C5b leads (via a number of steps involving C6, C7, C8, and C9, not shown) to formation of the membrane attack complex (MAC) that lyses bacteria/viruses through pore formation in their outer structures (see top right of figure). Host cells (see bottom half of figure) have a number of fluid-phase and membrane-anchored protective regulatory mechanisms. For example, FH binds to host cells via GAG or sialic acid and acts as a cofactor to enhance an enzyme, factor I (FI), to cleave C3b into an inactive product (iC3b), releasing a small peptide (C3f, not shown). Unlike C3b, C3bi is unable to participate in formation of C3 convertase and thus is unable to continue the alternative complement pathway. In addition, membrane cofactor protein (MCP), which is highly expressed on renal endothelium, also acts as a cofactor to promote FI cleavage of C3 into iC3b and C3f. Another membrane-anchored protein, thrombomodulin (TM), has at least three functions relating to complement regulation: first, TM enhances inactivation of C3b by FI in the presence of FH (bottom left of figure), as well as in the presence of C4b-binding protein (C4b-BP) (bottom middle of figure); second, TM catalyzes activation of thrombin activatable fibrinolysis inhibitor (TAFI) by thrombin (IIa), with activated TAFI (TAFIa) degrading C3a and C5a (bottom and middle right of figure); and third, IIa bound to TM is unable to cleave C5 into C5a and C5b (not shown). A transmembrane protein, decay-accelerating factor (DAF; not shown), dissociates C3bBb into C3b and Bb, but C3b can bind to another FB molecule if it is not proteolyzed by FI (acting with one of the two cofactors, FH or MCP) in the interim. The surface carbohydrate environment to which C3b is deposited determines the relative affinity of C3b for FH or FB. On host cells (bearing sialic acid and GAG), FH binds to C3b with a higher affinity than does FB. On microbial surfaces (lacking sialic acid and GAG), however, FB binds to C3b with a higher affinity than does FH, leading to amplified cleavage of C3. "Loss of function" mutations of several proteins of the alternative complement pathway produce uncontrolled activation of complement, as C3b is not degraded efficiently, leading to excess formation of C3 and C5 convertases. This predisposes affected patients to aHUS, with the percentages in parentheses indicating the frequency by which the particular complement protein abnormality has been identified in familial and sporadic aHUS: FH (15–30%), MCP (10–15%), FI (5–10%), and TM (5%). Autoantibodies against FH have also been reported as causing aHUS (6–11%). As well, certain "gain of function" mutations in FB (1–2%) and C3 (5–10%) also predispose patients to aHUS. Solid arrows indicate activation events; dashed arrows indicate inhibitory actions. Factors with heavy solid borders have enzymic activity

other disorders (e.g., SLE, HIV, *H. pylori*, hepatitis C, lymphoma, sarcoidosis) designates the ITP as "secondary." Approximately 60–70% of patients have detectable platelet-specific autoantibodies.

(a) First-line therapy of ITP includes IVIg or corticosteroids, although most patients will eventually relapse during corticosteroid tapering.

(b) Second-line therapy with splenectomy offers a good chance of cure (~70%) or meaningful platelet count improvement (10–20%), although late relapse is possible. An alternative to splenectomy includes anti-CD20 therapy (rituximab), a non-FDA-approved indication. In recent years, splenectomy has been performed less often for ITP.

(c) The thrombopoietin receptor agonists, eltrombopag (oral agent administered daily) and romiplostim (administered by weekly subcutaneous injection), have been considered second- or third-line therapy but can be used after initial treatment of steroids and IVIg. They are generally well tolerated and improve the platelet count in 60–80% of patients [29, 30]. For most patients, these drugs must be administered as ongoing maintenance therapy; however, up to 30% of patients may experience a remission even after therapy is discontinued [31, 32].

(d) Third-line therapies are numerous (danazol, mycophenylate, azathioprine, cyclosporine, combination chemotherapy, etc.), attesting to variable benefit and toxicity profiles. Non-splenectomized Rh(D)-positive patients can have platelet count increases for 1–3 weeks following treatment with anti-D, because antibody-coated red cells interfere with splenic platelet clearance. Overt warm IgG-induced hemolysis is a risk of anti-D, and this treatment is not recommended when patients have secondary ITP, are elderly, or have concomitant autoimmune hemolysis (Evan's syndrome) or a positive direct Coombs test (See Chap. 9).

(e) Emergency treatment of severe, symptomatic ITP includes high-dose corticosteroids and/or IVIg; platelet transfusions are usually restricted to patients with life-threatening bleeding since they do not last long (minutes to hours) in circulation. High-dose dexamethasone offers a more rapid rise in platelet count over standard-dose prednisone, but is no more likely to achieve a durable remission [33].

4. *Drug-induced autoimmune thrombocytopenia.* Rarely, drugs (e.g., levodopa, procainamide) cause ITP by triggering platelet-reactive autoantibodies or mimic ITP (gold) because slow elimination causes D-ITP to persist.

B. *Alloimmune thrombocytopenia.* Alloantigens are genetically determined molecular variations of proteins or carbohydrates that can be recognized immunologically by some normal individuals when exposed to the alloantigen(s) they lack, usually as a result of pregnancy, transfusion, or transplantation. Alloimmune thrombocytopenia results when antihuman platelet antigen (HPA) alloantibodies cause premature destruction of platelets via accelerated reticuloendothelial clearance. Five syndromes are recognized [34].

1. *Neonatal alloimmune thrombocytopenia (NAIT)* (see Chap. 34) is a potentially severe transient alloantibody-mediated thrombocytopenia that occurs in 1/2000 newborns. Passive placental transfer of anti-HPA-1a alloantibodies from an HPA-1b/b mother (2% of the population) is the most frequent cause; however, more than a dozen other platelet alloantigens have been implicated in NAIT, with some alloantigen systems (HPA-5a/b, -15a/b) causing less severe thrombocytopenia. Unlike hemolytic disease of the newborn, the first-born offspring is often affected by NAIT. A general rule: subsequent alloantigen-positive newborns evince thrombocytopenia *at least* as severe as their previously affected sibling(s). Treatment of unexpected NAIT involves transfusion of random or (preferably) compatible platelets, whereas prenatal management of an affected fetus involves specialized care through a tertiary fetomaternal unit.
2. *Posttransfusion purpura (PTP)* is a rare disorder characterized by severe thrombocytopenia (platelet count <20,000/μL) and mucocutaneous bleeding that begins 5–10 days after exposure to a blood product, usually at surgery (Fig. 15.3a). Most patients (>95%) are elderly parous females who are homozygous HPA-b/b, i.e., they form high-titer anti-HPA-1a alloantibodies that somehow destroy autologous platelets. "Random" platelet transfusions (usually bearing HPA-1a alloantigens) often trigger febrile or even anaphylactoid reactions. This transient life-threatening, hemorrhagic disorder (intracranial bleeding is the most common cause of death) usually is benefited by high-dose IVIg. Therapeutic plasma exchange can be considered if IVIg is ineffective.
3. *Passive alloimmune thrombocytopenia (PAT)* is a rare disorder characterized by the abrupt onset of thrombocytopenia within a few hours after transfusion of a blood product containing platelet-specific alloantibodies, most often anti-HPA-1a, which will destroy the transfusion recipient's platelets if they bear the target antigen. Implicated blood donors should no longer donate blood product.

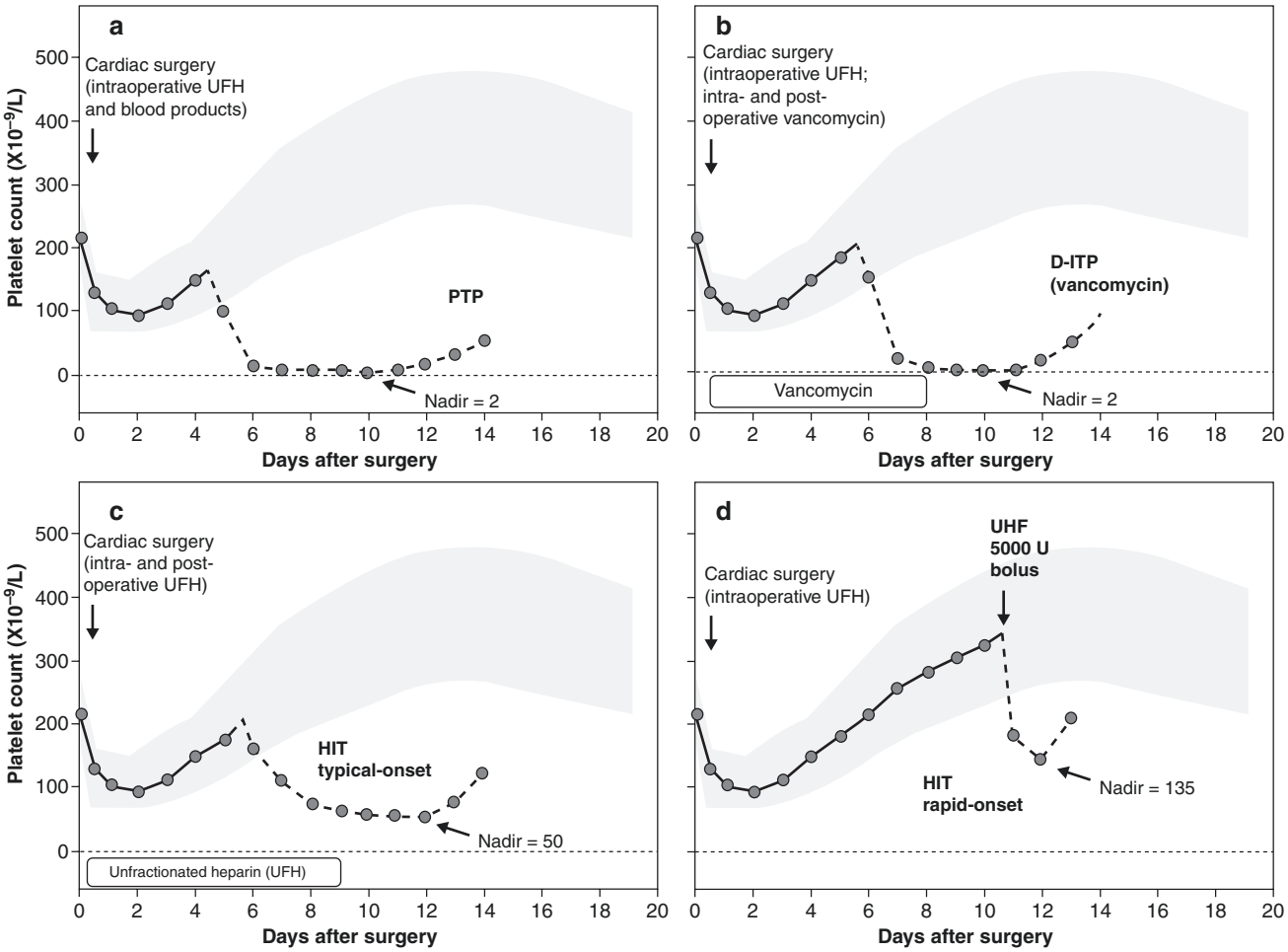

Fig. 15.3 Clinical features of four different explanations for postoperative thrombocytopenia. For each example, the "background" platelet count profile is as per Fig. 15.1 (post-cardiac surgery). (**a**) Posttransfusion purpura (PTP) is characterized by severe thrombocytopenia (platelet nadir usually <10,000/μL) that usually begins approximately 5–8 days after administration of blood product. (**b**) Drug-induced immune thrombocytopenia (D-ITP) is characterized by severe thrombocytopenia (platelet count nadir usually <20,000/μL) that usually begins approximately 5–10 days after beginning a new drug (vancomycin in the example shown). (**c**) Typical-onset heparin-induced thrombocytopenia (HIT) is characterized by mild, moderate, or severe thrombocytopenia (median platelet count 60,000/μL; 90% of patients have platelet count nadirs between 15,000 and 150,000/μL) that begins 5–10 days after beginning an immunizing course of heparin (the remaining patients have nadirs <15,000 or >150,000/μL); in the example shown, the immunizing exposure to heparin is intraoperative administration of unfractionated heparin (UFH) during cardiac surgery. Occasionally, an identical platelet count profile can be seen even when postoperative UFH is not administered; in this case, the patient is said to have "delayed-onset HIT." (**d**) Rapid-onset HIT is characterized by an abrupt decrease in platelet count after administering heparin. In the example shown, the platelet count fell immediately after a heparin bolus was given on postoperative day 18. HIT antibodies were already present at the time that the heparin bolus was given, as they were formed by the intraoperative exposure to heparin 18 days earlier at cardiac surgery. Sometimes, anaphylactoid reactions accompany rapid-onset HIT (see Box 15.2). Interestingly, because HIT antibodies are transient, a future heparin bolus given to the same patient 6 months later is very unlikely to be complicated by rapid-onset of thrombocytopenia

4. *Transplantation-associated alloimmune thrombocytopenia.* Rarely, thrombocytopenia that occurs weeks or even months following bone marrow transplantation (BMT) or solid organ transplantation can be caused by platelet-reactive alloantibodies generated either by residual host lymphoid cells against donor platelets (post-BMT) or from donor organ-derived "passenger" lymphocytes that destroy the organ recipient's platelets.
5. *Platelet transfusion refractoriness.* Platelet-specific alloantibodies occasionally contribute to platelet transfusion refractoriness (poor platelet count increments following platelet transfusion), but usually other mechanisms are operative (e.g., increased platelet consumption in very ill patients).

C. *Classic drug-induced immune thrombocytopenic purpura (D-ITP)*

1. *Quinine, etc.* Box 15.1 lists some of the drugs implicated in D-ITP. The typical picture is onset of severe thrombocytopenia (<20,000/μL) (Fig. 15.4) [35–38] that begins approximately 1 week after starting a new

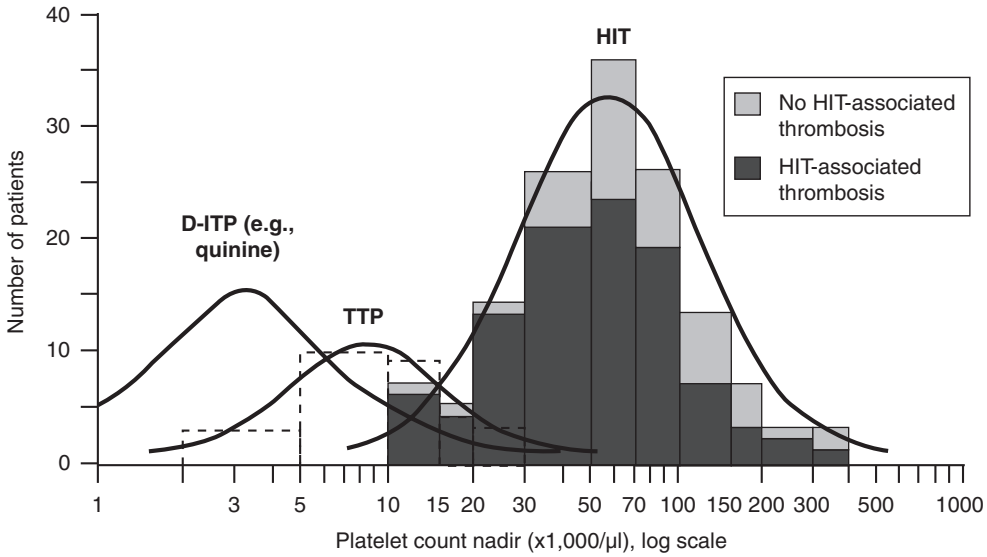

Fig. 15.4 Distribution of platelet count nadirs in three different thrombocytopenic syndromes: D-ITP (quinine) vs. TTP (with absent ADAMTS13 activity) and HIT (two subgroups: with and without thrombosis). (Reprinted, with modifications, with permission, from: [35])

drug known to cause this reaction (Fig. 15.3b). Petechiae are common; oral mucosal "blood blisters" and generalized GI or GU bleeding indicate more severe disease and risk of intracranial hemorrhage. Besides stopping potentially implicated drug(s), therapy includes one or more of platelet transfusions, high-dose IVIg, and corticosteroids. When an outpatient presents with severe thrombocytopenia, it is important to inquire specifically about *quinine*, a medication used to treat "leg cramps" but also a constituent of "tonic water" and a common cause of D-ITP [39].

2. *Atypical D-ITP secondary to GPIIb/IIIa antagonists.* Approximately 1% of patients develop severe thrombocytopenia (usually, <20,000/μL) within a few hours of receiving a GPIIb/IIIa antagonist (abciximab, eptifibatide, tirofiban), even with first-time exposure, due to preexisting, naturally occurring antibodies [40, 41].

D. *Heparin-induced Thrombocytopenia (HIT)*

HIT is an important adverse drug reaction: it is relatively common (~1% to 3% of patients receiving >1 week of UFH for postoperative prophylaxis) and strongly associated with life- and limb-threatening thrombosis [42, 43]. It can also occur after low-molecular-weight heparin administration [1] and rarely has been reported with fondaparinux [44].

1. *Pathophysiology.* HIT is caused by IgG that binds via Fab to multimolecular complexes of platelet factor 4 (PF4) bound to heparin; the Fc moieties bind to platelet Fc receptors, producing strong platelet activation. Formation of procoagulant platelet-derived microparticles, and activation of endothelium and monocytes, contributes a prothrombotic state (in vivo thrombin generation).
2. *Clinical Features.* An otherwise unexplained thrombocytopenia or a 50% drop in a normal platelet count bearing a temporal relation to a preceding immunizing exposure to UFH (usually given intra- or postoperatively) strongly suggests HIT [1], as this disorder is seen 10–15× more often with UFH compared with LMWH [45], and 3–5× more often in surgical versus medical patients [46]. The median platelet count nadir is ~60,000/μL; for 85–90% of patients, the platelet count nadir ranges between 20,000 and 150,000/μL (Fig. 15.4) [35, 42]. At least half of all patients with proven HIT develop clinically evident sequelae, usually symptomatic thrombosis (Box 15.2) [1, 2].
   (a) Typical-onset HIT (~65% of cases) refers to a platelet count fall that begins 5–10 days after an immunizing exposure to heparin, and while the patient continues to receive heparin (Fig. 15.3c) [47].
   (b) Rapid-onset HIT (~25% of cases) refers to an abrupt drop in platelet count upon starting heparin (Fig. 15.3d); almost invariably, patients were exposed to heparin within the preceding several weeks, thus explaining why thrombocytopenia begins abruptly when heparin is recommenced [47].
   (c) *Autoimmune HIT* is characterized by HIT that begins, worsens, or persists in the absence of heparin (or with small amounts of heparin, e.g., heparin "flushes") [48]. Patients have high levels of anti-PF4/heparin antibodies that are able to activate platelets in vitro even in the absence of heparin. A number of syndromes are reported:
      (i) Delayed-onset HIT (~10% of cases) resembles typical-onset HIT, except that patients are no longer receiving heparin when HIT and HIT-associated thrombosis begins or worsens

[49]. Sometimes, the thrombocytopenia can persist for several weeks despite absence of ongoing heparin ("persisting HIT").

(ii) "Flush" heparin HIT (<1% of cases). Rarely, HIT occurs when a patient's only heparin exposure is small doses ("flushes") used for maintenance of intravascular catheters [50].

(iii) Spontaneous HIT syndrome (<1% of cases). Rarely, a syndrome clinically and serologically identical to HIT occurs in the absence of any preceding heparin exposure. Preceding infection or surgery (especially, knee replacement surgery) is believed to trigger "spontaneous" HIT antibodies [51].

(iv) Fondaparinux-associated HIT (<1% of cases) [44]. Rarely, fondaparinux is implicated as a de novo trigger of HIT. Although these patients' HIT antibodies characteristically activate platelets in the absence of heparin, some also exhibit increased platelet activation in the presence of fondaparinux.

3. *Laboratory testing for HIT antibodies.* Anti-PF4/heparin immunoassays (e.g., enzyme immunoassay [EIA]) and washed platelet activation assays (e.g., platelet serotonin-release assay) detect HIT antibodies. Approximately half of EIA+ patients with clinically suspected HIT do *not* have this diagnosis, because (more specific) platelet activation assays are negative, and other explanations for thrombocytopenia are evident [52]. Although the sensitive and specific serotonin release assay (SRA) is performed by only a few laboratories, the assay is nonetheless available on a referral basis. In the SRA (a "washed" platelet assay), platelets obtained from normal "pedigree" donors (known to respond well to HIT sera) are labeled with ^{14}C-serotonin and washed in buffer. A positive test is indicated when (heat-inactivated) serum from a patient with HIT causes strong platelet activation (substantial release of ^{14}C-serotonin from the washed donor platelets) in the presence of pharmacologic concentrations of heparin (0.1–0.3 U/mL) but not at high concentrations of heparin (100 U/mL). The "stronger" a positive EIA result (i.e., higher optical density [OD] units), the more likely it is the patient has platelet-activating antibodies and true HIT [53]. For example, a positive PF4/heparin EIA (normal cut-off, 0.4 OD units) with OD >2.0 units has a 90% probability of predicting for a positive SRA, whereas a weak-positive EIA result (0.4–1.0 OD units) has only a 5% probability of a positive SRA. Such a weak-positive EIA should therefore be taken as evidence *against* the diagnosis of HIT.
4. *Treatment.* For patients with serologically confirmed or strongly suspected HIT, whether or not complicated by thrombosis, treatment involves stopping heparin and giving an alternative, non-heparin anticoagulant, such as a direct thrombin inhibitor (argatroban, bivalirudin) or an antithrombin-dependent, factor Xa inhibitor (danaparoid [not available in the USA], fondaparinux [54]). There is also convincing evidence that direct oral anticoagulants (e.g., rivaroxaban, apixaban) are effective anticoagulants for HIT [55]. It is crucial to avoid or postpone warfarin therapy pending substantial recovery of HIT (platelet count >150,000/μL) since vitamin K antagonism is *the* major factor that explains HIT-associated limb amputation due to venous limb gangrene (limb ischemia in the setting of deep vein thrombosis (DVT) and with palpable/Doppler-identifiable pulses) [4, 56]. A supratherapeutic INR (>3.5) is a characteristic feature of warfarin-associated venous limb gangrene complicating HIT and represents a surrogate marker for severe protein C depletion. Progressive microvascular thrombosis results because warfarin fails to inhibit thrombin generation in HIT but results in failure to downregulate thrombin due to protein C depletion. Routine duplex ultrasonography of four limbs of patients with HIT is appropriate given the high frequency (~50%) of DVT in this patient population [2].
5. *Repeat heparin exposure.* HIT antibodies are transient (median time to non-detectability, 50–80 days depending upon the assay performed), are not invariably triggered by repeat heparin exposure, and (if regenerated) require at least 5 days to reach significant levels [47, 57]. Accordingly, for patients with a previous history of HIT, repeat heparin exposure is the favored option for intraoperative anticoagulation when such patients need cardiac or vascular surgery [57].

Pseudothrombocytopenia

Naturally occurring antibodies that cause ex vivo platelet agglutination (i.e., after collection of blood into tubes containing anticoagulant) can lead to a false diagnosis of thrombocytopenia because platelets within "clumps" are not counted by the electronic particle counter. This is why inspection of the blood film is crucial before a laboratory reports a first occurrence of thrombocytopenia in a patient. Pseudothrombocytopenia is clinically insignificant, except when it leads to inappropriate treatment because of wrongly suspected thrombocytopenia or makes it difficult to obtain an accurate platelet count in an affected patient who also develops true thrombocytopenia.

A. *EDTA-induced pseudothrombocytopenia* is the most common type of pseudothrombocytopenia and results from antibody-induced agglutination in the presence of

the chelating anticoagulant, ethylenediaminetetraacetic acid (EDTA) [58]. Often, a normal platelet count result will be obtained if the blood is collected into another anticoagulant (sodium citrate, heparin) and/or collected into prewarmed tubes maintained at 37 °C before platelet enumeration. Approximately one-quarter of patients with apparent GPIIb/IIIa antagonist-induced acute thrombocytopenia have pseudothrombocytopenia.

B. *Platelet satellitism* is the phenomenon of platelet rosetting around neutrophils in EDTA-anticoagulated blood and is caused by antibodies that make platelets adhere to neutrophils.

C. *Miscellaneous causes of pseudothrombocytopenia* include platelet cold agglutinins and paraproteinemias. As noted earlier, giant platelets as seen in MYH9-associated macrothrombocytopenia may lead to a falsely low estimate of the platelet count (see Chap. 13).

Box 15.1 Drug-Induced-ITP: Implicated Drugs (Partial List)

Typical (relatively common): quinine/quinidine, sulfa antibiotics, carbamazepine, vancomycin, piperacillin, and tazobactam

Typical (less common): acetaminophen, actinomycin, amiodarone, amitriptyline, amoxicillin/ampicillin/piperacillin/nafcillin, cephalosporins (cefazolin, ceftazidime, ceftriaxone), celecoxib, ciprofloxacin, esomeprazole, ethambutol, fexofenadine, fentanyl, fusidic acid, furosemide, gold salts, haloperidol, ibuprofen, irinotecan, levofloxacin, metronidazole, naproxen, oxaliplatin, phenytoin, propoxyphene, propranolol, ranitidine, rifampin, simvastatin, suramin, trimethoprim, valproic acid

Atypical[a]: glycoprotein IIb/IIIa antagonists: abciximab, eptifibatide, tirofiban.

[a]Usually occurs in patients who have never been previously exposed to a GPIIb/IIIa antagonist

Box 15.2 Clinical Sequelae of HIT

Thrombosis

Venous: DVT (lower and upper limb[a]), PE, adrenal vein thrombosis (manifests as adrenal hemorrhage), cerebral venous (dural sinus) thrombosis, mesenteric or portal vein thrombosis

Arterial: limb artery, cerebral, myocardial, brachial, mesenteric, renal, others

Intracardiac: intra-atrial, intraventricular

Microvascular: usually warfarin-associated (venous limb gangrene); sometimes, overt (decompensated) DIC alone explains microvascular thrombosis

Heparin-induced skin lesions (at heparin injection sites): necrotizing and non-necrotizing

Anaphylactoid reactions (post-intravenous heparin bolus): inflammatory (fever, chills), cardiorespiratory (chest pain/tightness, tachycardia, hypertension, cardiac arrest, dyspnea, tachypnea, respiratory arrest), gastrointestinal (diarrhea), neurologic (transient global amnesia)

[a]Upper limb DVT in HIT is strongly associated with central venous catheter use

DIC disseminated intravascular coagulation, *DVT* deep vein thrombosis, *PE* pulmonary embolism

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Thrombosis

16

Sargam Kapoor, Mukesh K. Jain, and Lalitha Nayak

Overview

Thromboembolism is a leading cause of morbidity and mortality globally. Survival after VTE (venous thromboembolism including deep venous thrombosis and pulmonary embolism) is worse than the expected survival in age- and sex-matched controls [1, 2]. Moreover, the risk of death after a pulmonary embolism (PE) is 18-fold higher than after a deep vein thrombosis (DVT) [3]. VTE also poses a significant burden globally in terms of disability-adjusted life years (DALYs) lost in low-, middle-, and high-income countries [4]. In the year 2010, one in four deaths worldwide was attributed to arterial thromboembolic conditions (12.9 million deaths from arterial thromboembolic conditions compared to eight million deaths from cancer) [5]. These arterial thromboses primarily include myocardial infarction, ischemic strokes, and limb ischemia, whereas deep vein thrombosis and pulmonary embolism comprise the bulk of venous thrombosis.

In 1856, Virchow described the three causes of thrombosis: blood hypercoagulability, stasis, and vessel wall abnormalities (Virchow's triad). Blood components (including blood cells and plasma proteins) are the best studied of this triad, and the importance of alterations in pro- and anticoagulant proteins in the genesis of thrombosis is well established. In addition, the vessel wall is a major contributor to thrombosis risk and prevention. Its basic nature is antithrombotic, but with injury and inflammation, it turns into a prothrombotic organ. The vessel wall provides adhesion receptors that enable recruitment of leukocytes and platelets to sites of vascular injury and dysfunction. Upon injury, endothelium expresses tissue factor and exposes vascular smooth muscle tissue factor that is constitutively present. Additionally, vascular endothelial cells are constantly subjected to mechanical shear stress imposed by blood flow. Fluid shear stress itself through oscillatory and turbulent flow regulates vascular biology and pathology by ordering changes in protein expression via induction of vascular transcription factors. In this chapter we provide an overview of arterial and venous thrombosis. It will then focus on the pathogenesis of the complex and dynamic processes underlying venous and arterial thrombosis and the conditions that predispose to them. It will highlight disorders and pathogenetic markers to both. It aims to convey the concept that venous and arterial thrombosis forms a continuum with some features unique to each and others common to both.

Key Concepts

1. In the normal physiologic state, there is a tightly regulated balance between the procoagulant factors (that cause thrombus formation), anticoagulant factors (that limit thrombus formation), and the fibrinolytic mechanisms (that facilitate clot lysis). Several inherited and acquired risk factors can disrupt this delicate balance and increase thrombotic risk.
2. Arterial and venous thromboses have been known to have distinct pathogenetic mechanisms that differ in terms of the relative contributions of each of these factors [6]. However, recent data link VTE and atherosclerosis [7, 8].
3. The genesis of thrombosis is multifactorial and is due to vascular, cellular, protein, or rheological defects or a combination of these factors.
4. Therapy for thrombosis is directed toward lysis of clots, prevention of clot extension, and the occurrence of future thrombotic events.

Pathogenetic Features

The occurrence of venous and arterial thrombosis in a patient in all cases can be ascribed to the summation of risk factors in the subject. Broadly, risk factors for thrombosis are

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categorized into inherited and acquired (discussed in detail below). Broadly speaking, one or more risk factors are identified in ≥80% patients with VTE [9]. Combining the currently available data, we are able to recognize inherited thrombophilia as a cause for venous thrombosis in 24–37% of patients with venous thrombosis compared to about 10% in controls [10–12]. Most individuals who present with thrombosis have one or more of the following features at the time of presentation, fulfilling most or all elements of the Virchow's triad.

1. Vascular endothelial and smooth muscle dysfunction
2. Stasis of blood
3. Platelet and leukocyte activation
4. Activation of coagulation proteins

Red (Fibrin) Versus White (Platelet) Clots

Although both venous and arterial thrombi contain platelets and fibrin, the relative proportion of each and the structure of a thrombus vary as to the vessel in which it is formed.

1. In low-flow vessels (e.g., veins), the initial platelet plug, which may have started the thrombus, is often not detected. These clots result from the accumulation of red blood cells in fibrin strands and are called red thrombus.
2. In a high-flow arterial vessel, platelet-rich thrombi are seen. Macroscopic aggregates of platelets have a white appearance (Fig. 16.1).

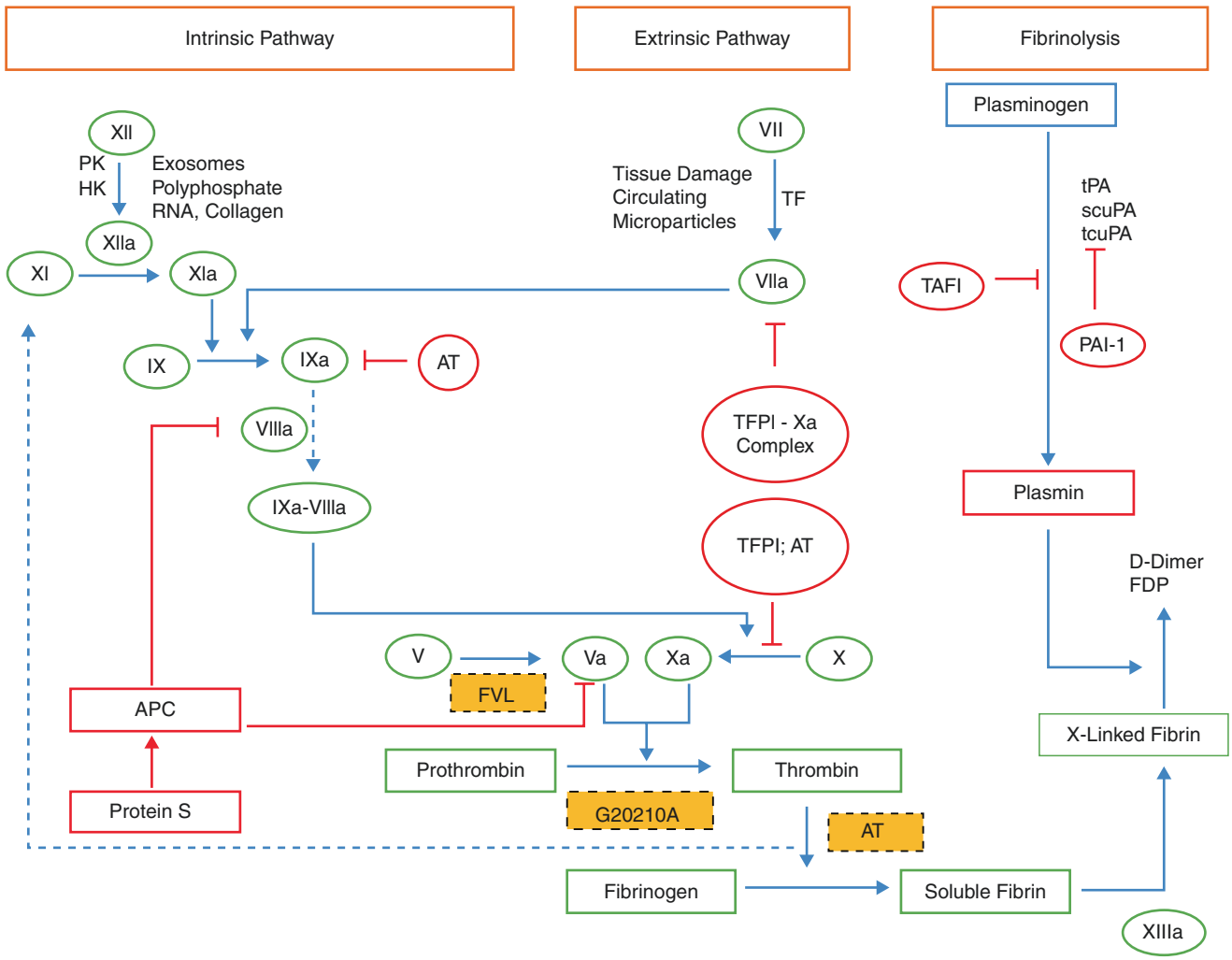

Fig. 16.1 Biochemistry of venous thrombosis. This figure is a juxtapositioning of the proteins involved in the coagulation (green outlines), anticoagulation (red outlines), and the fibrinolytic (blue outlines) systems. PK is prekallikrein; HK is high molecular weight kininogen; coagulation factors XII, XI, IX, X, VII, V, and XIII are represented by their roman numeral alone. The presence of an "a" after the roman numeral represents an "activated" protein. TF is an abbreviation for tissue factor; FVL is an abbreviation for factor V Leiden; AT is antithrombin deficiency; G20210A is the prothrombin gene mutation; scuPA is single-chain urokinase plasminogen activator; tcuPA is two-chain urokinase plasminogen activator; tPA is tissue plasminogen activator; TFPI is tissue factor pathway inhibitor; and TAFI is thrombin-activable fibrinolysis inhibitor (procarboxypeptidase B)

Venous Thrombosis

Introduction

Venous thrombi form in low blood flow states. A venous clot is composed of two regions, a red cell-rich fibrin clot that lies adjacent to the endothelium and lines of platelet-rich white clot, also known as lines of Zahn that lie further inside the clot. Leukocytes can be seen lining the vessel wall in experimental restriction to flow venous thrombosis as well as being trapped in the fibrin of the red clot. An experimental model to create venous thrombosis requires the following: vessel injury, reduced blood flow, inhibition of an anticoagulant system, and induction of an inflammatory response. These models require the recruitment of leukocytes or leukocyte microparticles for the initiation of thrombus formation. Thus, vein wall injury allows exposure of procoagulant membrane surfaces and adhesive molecules so that both leukocytes and platelets are recruited to the site of injury and initiate the thrombotic process.

Pathogenesis

The hemostatic response to vascular injury is a tightly regulated and complex process and was believed to include three important components: (1) normal functioning platelets; (2) von Willebrand factor (vWF), the protein that mediates adhesion of platelets to the damaged endothelium in the presence of high shear; and (3) constriction of the blood vessel in response to injury. This results in the formation of a platelet plug and arrests bleeding following vessel injury. This hypothesis is the platelet plug notion of so-called primary hemostasis.

The introduction of genetically modified mice coupled with hemostasis/thrombosis models has led to novel insights into the complex process of hemostasis. Recent advances in intravital microscopy imaging techniques suggest that hemostasis involves a dynamic and complex interaction between coagulation factors, the vessel wall, and the cellular elements in the blood [13]. These findings also indicate that adequate amounts of thrombin (≥10 nM) are formed in the absence of robust platelet accumulation indicating that other biological surfaces are sufficient to support coagulation reactions at the site of vascular injury. These biological surfaces include the endothelial cells and leukocytes. Thus, endothelial dysfunction or damage can form a procoagulant surface resulting in thrombin generation. Although vascular injury is followed by a rapid formation of a platelet aggregate, intravital imaging studies demonstrate that platelet activation at the site of vascular injury is heterogeneous. Thus, a subpopulation of platelets spatially localized adjacent to the site of injury are P-selectin positive, stably adherent, and generally referred to as the "core" of the thrombus. This nidus of a thrombus is overlaid by a "shell" of less activated and loosely adherent platelets. During the process of hemostasis, blood flow, platelet activation, and the coagulation system determine the generation of the platelet-fibrin thrombi at the site of vessel wall injury. There are numerous factors in the vessel wall that can trigger this process, including collagen and tissue factor (TF). Murine models of thrombosis suggest that the generation of a thrombus is determined by the relative availability of each of these platelet-adhesive (e.g., collagen) and coagulation-triggering (e.g., TF) factors. Under conditions of high shear blood flow where decreased exposure to collagen limits platelet adhesion, platelet-dependent fibrin formation and platelet deposition are decreased. In this situation, the contribution of thrombin to the generation of a thrombus becomes relatively more important, and fibrin formation is high in the top region of the thrombus. On the contrary, under conditions of high collagen exposure and increased platelet adhesion, fibrin deposition is noted primarily at the base of the thrombus [14].

Unlike arterial thrombi, platelets have a lessor role in the pathogenesis of venous thrombosis where they typically accumulate at the head of the venous thrombus. Tissue factor from overt vessel damage or from microparticle- or exosome-derived material from blood and other cells (e.g., circulating cancer cells) combines with factor VIIa and initiates the coagulation cascade. Increase in coagulation factors or defects in the anticoagulant mechanisms (heparin-antithrombin, activated protein C, and tissue factor pathway inhibitor) increase risk and facilitate thrombosis (see below). Thrombin generation from this process activates platelets by cleaving PAR4/PAR1 (protease-activated receptors) on the platelet surface that in turn activates other platelets and amplifies thrombus formation. Vessel wall collagen activation of platelet GPVI is another independent mechanism contributing to clot formation.

Injury to the vessel wall occurs through various mechanisms. Although overt damage to the endothelium is not found in venous thrombosis, stimuli for endothelial cell dysfunction arise from many sources. Hypoxia occurring in the low-flow, static pockets of venous valves has been proposed as a stimulus. Infections (viral, bacterial, and rickettsial) that change the nature of the venous endothelium are other inciting etiologies. Inflammatory cytokines have similar effects. Further, endothelial denudation may occur with the use of indwelling central venous catheters that are a mechanical example commonly induced in medical care. There are four phases that characterize vessel injury.

1. *Initial phase*: leukocyte recruitment and platelet thrombus formation in an area of low flow
2. *Acute phase*: active fibrin clot formation, initiation of a greater inflammatory response

3. *Intermediate phase*: thrombus limitation and reduction of the inflammatory response
4. *Chronic phase*: reabsorption and recanalization of the clot

Risk Factors

Thrombosis is the result of a summation of acquired and genetic risk factors. Usually, an acquired risk factor causes a second hit over an existing or baseline thrombotic risk resulting in formation of a clot. Major complications of venous thrombosis include disabling post-thrombotic syndrome, pulmonary hypertension, and death secondary to pulmonary embolism.

Inherited Risk Factors for Venous Thrombosis

These risk factors are divided into (i) common and high risk and (ii) other molecular etiologies.

i. *Inherited high-risk factors for venous thrombosis* (Table 16.1)

(a) *Factor V Leiden* (FVL)

FVL is the most common inherited hypercoagulable state and accounts for ≥20% of patients with first-time VTE. Its prevalence is 5–10% among Caucasians and is rare in other ethnic groups although it is found in areas of the world where European traders mixed with local population. The FVL mutation is a point mutation that results in the substitution of arginine to glutamine at position 506 in the factor V gene. Activated protein C (APC) is a potent anticoagulant that works with its cofactor protein S to cleave the activated forms of factors V and VIII. The mutated factor V lacks a cleavage site and is thereby less susceptible to inactivation by APC. Inheritance is autosomal dominant. FVL heterozygosity leads to a three- to sevenfold increased thrombotic risk, whereas homozygosity causes an 80-fold increased risk compared to normal individuals [15]. This risk is further increased with oral contraceptive use (48-fold), pregnancy, protein S deficiency, and increasing age. Heterozygosity for FVL does not affect population mortality [16, 17]. There are no data available on life expectancy in individuals homozygous for FVL mutation. FVL is a higher risk for thrombosis in pregnancy that is protein C or S deficient.

(b) *Prothrombin 20210 mutation*

The prothrombin gene mutation is the second most common inherited thrombophilia (2–4% of the population) and is predominantly found in the southern European population. Like the FVL mutation, it is rare in non-Caucasians. It is caused by a point mutation in the factor II gene (prothrombin gene) at position 20210A (3′ UT) that results in higher levels of circulating factor II. It is associated with 6–8% of thrombosis. Inheritance is in an autosomal dominant fashion. Heterozygosity of this mutation confers a threefold risk of first-time VTE. Homozygosity of this mutation is rare, and thus the risk is not known. Patients with double heterozygous mutations for both FVL and prothrombin 20210A show a much higher risk (20-fold) of thrombosis as compared to single mutations.

(c) *Protein C deficiency*

The incidence of clinically significant or symptomatic inherited protein C deficiency is estimated at 1 in 20,000, whereas asymptomatic protein C deficiency has been reported in 1 in 200–500 healthy individuals [18]. There is no racial predilection for its deficiency. Protein C is a vitamin K-dependent coagulation protein. The activated form (APC) has an important role in inactivating factors Va and VIIIa, thereby regulating thrombin generation. Thus, deficiency of this protein results in a procoagulant state. Most genetic protein C deficiencies result in a quantitative defect (type 1), while 15% cause a qualitative defect (type 2). The annual risk of thrombosis ranges 0.5–2.5%, and the lifetime risk is 75%. In addition to the risk of VTE, protein C deficiency is also associated with risk of arterial events (stroke) in the young. Homozygous or compound heterozygous forms are rare (1 in one million) and result in severe deficiency. Inheritance is in an autosomal dominant fashion.

There are a number of clinical syndromes associated with protein C deficiency. These include:

(i) Venous thrombosis (heterozygous defect)
(ii) A form of warfarin-induced skin necrosis (heterozygous defect)
(iii) Neonatal purpura fulminans (homozygous or compound heterozygous defect)
(iv) Disseminated intravascular coagulation (both)

Deficiency is acquired in patients secondary to underproduction (warfarin, liver disease, prematurity) or overconsumption (disseminated intravascular coagulation (DIC), acute VTE, heparin-induced thrombocytopenia, and thrombosis and antiphospholipid antibody syndrome). Thus, protein C levels should be considered in clinical states where one may be having accelerated coagulation.

Table 16.1 Epidemiology of inherited procoagulant states

Defect	Incidence in population	% of patients with procoagulant states
Factor V Leiden	5–10%	20–60%
Prothrombin gene mutation	2–4%	6–8%
Homocysteine	–	10%
Protein C deficiency	1:200	<5%
Protein S deficiency	–	<5%
Antithrombin deficiency	1:2000–5000	<1%
Dysfibrinogenemia	Unknown	1–2%

(d) *Protein S deficiency*

Protein S is also a vitamin K-dependent protein that exists in two forms: free form (active, 40%) and the bound form (with C4b-binding protein, 60%). In plasma, functional protein S has to be free or unbound to C4b-binding protein. Protein S lacks enzymatic activity and serves as a cofactor of APC for the proteolytic degradation of factors Va and VIIIa. It also enhances the profibrinolytic effects of APC by neutralization of plasminogen activator inhibitor. Protein S deficiency is classified as type 1 (decreased levels of both total and free protein S antigen), type 2 (decreased cofactor activity but normal total and free protein S antigen), and type 3 (decreased levels of free protein S antigen only). Prevalence of this disorder is largely unknown due to its rarity and difficulty in correct diagnosis. Inheritance is in autosomal dominant fashion. Fifty percent of individuals with this deficiency develop VTE by 55 years of age [19, 20]. Earlier studies suggested that the set of individuals with protein S deficiency is much larger than the set of individuals with thrombosis suggesting that the thrombosis risk of protein S deficiency is unclear. However, as one ages, the presence of other thrombosis risk factors may summate with protein S deficiency.

Acquired forms of protein S deficiency are recognized. For example, protein S values are low in pregnancy and estrogen therapy where there is dilution or reduced synthesis, liver disease with reduced synthesis of this vitamin K protein, warfarin therapy with reduced and abnormal synthesis, nephrotic syndrome where the protein is lost due to glomerular leakage, HIV infection, and DIC due to consumption. Additionally, the C4-binding protein that binds protein S increases in inflammatory states to reduce the amount of freely circulating protein [21]. The clinical significance of acquired protein S deficiency may be different from the inherited form, but this assessment is not confirmed.

(e) *Antithrombin deficiency*

Antithrombin (AT) is a serine protease inhibitor or SERPIN that primarily inactivates thrombin and factor Xa. It also inhibits factors VIIa, IXa, XIa, XIIa, tPA (tissue plasminogen activator), plasmin, and kallikrein. Its deficiency is uncommon and is inherited in an autosomal dominant manner. It is the first known inherited thrombophilia that was described in 1965. Most cases are heterozygous as homozygosity is incompatible with mammalian life. This defect is a severe prothrombotic state that carries a 50% lifetime risk of developing a VTE and, when recognized, requires anticoagulation therapy for life. Acquired deficiency occurs due to decreased synthesis (cirrhosis), loss (protein-losing enteropathy, nephrosis), and increased consumption (sepsis/DIC, cardiopulmonary bypass, thrombotic microangiopathies, large hematomas/tumors, ECMO) or can be drug induced (heparin consumption in anticoagulation, L-asparaginase therapy-proteolysis).

It is important to know that the anticoagulant effect of heparin, low molecular weight heparin, and fondaparinux is mediated through an antithrombin-dependent mechanism.

ii. *Inherited low-risk factors associated with venous thrombosis*

(a) *Homocysteine*

It is described in detail below in section on arterial thrombosis. Hyperhomocysteinemia also confers a risk of venous thrombosis.

(b) *Disorders of Fibrinogen*

Fibrinogen functions as a precursor of the fibrin polymer in the coagulation system. Disorders involving fibrinogen include congenital afibrinogenemia, hypofibrinogenemia, dysfibrinogenemia, and hypodysfibrinogenemias (decreased quantity or defective quality of fibrinogen). These disorders can be inherited or acquired. Although they are generally associated with bleeding manifestations, certain forms have thromboembolic complications. The prothrombotic forms of dysfibrinogenemia are congenital (up to 30% of individuals may be affected) [22]. Congenital dysfibrinogenemia is a rare cause of thrombophilia accounting for 0.8% of patients with venous thrombosis. There are reports of both venous and arterial thromboembolic complications with congenital afibrinogenemia [23]. The mechanisms of thrombophilia in these conditions have yet to be elucidated. Thrombosis risk for fibrinogen is mostly associated with the density and thickness of the fibrin strands. Additionally, the concentration of fibrinogen itself is a factor contributing to thrombus since a structurally normal fibrinogen at 600 mg/dl confers a greater risk for venous thrombosis than a concentration at 300 mg/dl. Finally, fibrinogen function itself due to polymorphism may be additionally contributory. A fibrinogen that is more adhesive to platelets or less subject to fibrinolysis would shift the balance to a prothrombotic state.

(c) *Lipoprotein(a) or Lp(a)*

Lp(a) consists of apolipoprotein(a) that is linked to apolipoprotein B-100 by a disulfide bond. Lp(a) is structurally identical to kringle 4 of plasminogen. Lp(a) competes plasminogen binding to fibrin and vessel wall leading to impaired fibrinolysis. Lp(a) is also proatherogenic since it delivers apolipoprotein(a) to the vessel wall contributing to atherosclerosis. Elevated lipoprotein(a) also is a known risk factor for ischemic cardiovascular and cerebrovascular disease. In 2000, Lp(a) was established as an independent risk factor for VTE as well. Increased Lp(a) was found in 20% of patients with thrombosis and 7% of healthy controls in a study with 700 patients [24].

(d) *Elevation of coagulation factors (II, VII, VIII, IX, and XI), von Willebrand factor (vWF), plasminogen activator inhibitor-1 (PAI-1), and thrombin-activable fibrinolysis inhibitor (TAFI)*

Increased plasma levels of the above factors in epidemiologic studies have been associated with increased thrombosis in numerous studies. What this information shows is that if there is increased substrate for clot formation, it drives the reaction toward forming thrombosis. If there is reduced fibrinolysis due to excessive inhibitors to plasmin formation, the balance also will be pushed toward thrombosis. Precise values of coagulation factors in individual cases are not useful, but this information helps one understand clinical conditions like Cushing syndrome where hypercortisolism drives up the levels of blood coagulation factors leading to a 30–40% increase in venous thromboembolism in these patients.What.

Acquired Risk Factors for Venous Thrombosis (Table 16.2)

Age

The incidence of both idiopathic and secondary VTE increases markedly with age for both men and women. While the rate of venous thrombosis is about 1 per 10,000 person years before the fourth decade of life, it rises rapidly after age 45 and approaches 8 per 1000 person years by age 80 [26]. There is also a steeper rise in the incidence of PE as compared to DVT with aging. The reasons for an increased risk for thrombosis with age are not completely understood, but it may be secondary to comorbid conditions that predispose to thrombosis, increases in coagulation proteins, or a combination of these factors.

Trauma, Surgery, and Immobilization

Minor injury in the legs is strongly associated with VTE, whereas similar injuries to other parts of the body are not. Thrombotic risk is greatly increased during surgery especially orthopedic, vascular, brain, spinal, and cancer surgery. The risk depends upon the nature of the surgery and the duration of time that the patient is under anesthesia. Major orthopedic surgery involving the lower extremity is a major risk factor for VTE that without prophylaxis ranges from 40% to 60% within the first 2 weeks of surgery. Despite thromboprophylaxis with subcutaneous low molecular weight heparin during hospital course, total hip and knee replacement are still associated with symptomatic VTE in 1–3% of patients [27, 28]. The mechanisms for thrombosis in these patients are a combination of stasis and local accumulation of tissue factor. The risk of DVT in other surgeries follows accordingly: abdominothoracic surgery (14–35%); urologic surgery, transurethral resection (7%) and suprapubic prostatectomy (35%); and gynecologic surgery, vaginal hysterectomy (7%) and total abdominal hysterectomy (27%).

Venous stasis associated with bed rest, trauma, or neurologic disease is an important risk factor for VTE. Prolonged travel (car, train, or airplane, e.g., >4 h) also confers a two- to fourfold risk of VTE. "Economy-class syndrome" is the entity of VTE after a prolonged airplane flight. This event is amplified if the subject is on oral contraceptives and has FVL.

Table 16.2 Independent risk factors for deep vein thrombosis or pulmonary embolism [25]

Baseline characteristics	Odds ratio	95% CI
Body mass index	1.08	1.05–1.11
Major surgery	18.95	9.22–38.97
Hospitalization for acute medical illness	5.07	3.12–8.23
Nursing home confinement	4.63	2.77–7.74
Trauma/fracture	4.56	2.46–8.46
Active cancer	14.64	7.73–27.73
Neurologic disease with leg paresis	6.10	1.97–18.89
Pregnancy or postpartum	4.24	1.30–13.84
Oral contraceptives	4.03	1.83–8.89
Estrogen alone	1.81	1.06–3.09
Noncontraceptive estrogen plus progestin	2.53	1.38–4.63

Cardiac Disease

Patients with unprovoked VTE have a higher prevalence of atherosclerosis than those with secondary VTE [7]. Additionally, the long-term incidence of cardiovascular events is higher in patients with idiopathic VTE than those with provoked VTE. These studies support the postulate that VTE may be the first symptomatic cardiovascular event. Further, heart failure (particularly right HF) confers a hypercoagulable state that can be associated with intracardiac thrombi and deep vein thrombosis (DVT) [29].

Obesity/Metabolic Syndrome

The metabolic syndrome is frequently accompanied by a prothrombotic milieu evidenced by increased plasma levels of PAI-1, TAFI, and vWF, coagulation factor proteins (VIII, VII, and XIII), and fibrinogen. Metabolic syndrome is also associated with endothelial dysfunction (decreased nitric oxide and prostacyclin) and increased platelet reactivity. Activation of the hemostatic system in this situation is mainly attributed to effect of pro-inflammatory and proatherogenic mediators released by adipose cells and to chronic effects of hyperglycemia. Consistently, obesity (BMI> 30) increases the risk of thrombosis twofold, when adjusted for age and sex [30].

Inflammatory Bowel Disease (IBD)

Patients with IBD have an increased risk of thromboembolism, both arterial and venous [31]. Although the exact etiology is not known, it is thought that multiple acquired and inherited factors interact to generate a prothrombotic milieu.

Commonly we observe that the inflammatory state elevates C4B-binding protein producing an acquired protein S deficiency. IBD per se is a risk factor for thrombosis since other chronic inflammatory disorders, e.g., rheumatoid arthritis or chronic bowel diseases, e.g., celiac disease, are not associated with an increased VTE risk.

Cancer

Fifteen to 20% of VTEs occur in patients with cancer [32]. Alternatively, 4–20% of patients with cancer will have a thrombotic event [33]. Armand Trousseau first described the connection between occult malignancy and thrombosis in 1865. Later in 1867, he himself developed left arm phlebitis and diagnosed himself with gastric cancer of which he died 6 months later. The incidence of occult malignancy in patients with idiopathic thrombosis varies from 0.5% to 5.8%. However, there is a fourfold increase in the risk of thrombosis with cancer. Chemotherapeutic agents further increase this risk. This risk is attributed to the increased expression of tissue factor by tumor cells. In addition, there is increased production of microparticles [34] and inflammatory cytokines that promote clotting and tumor growth (via upregulation of VEGF) [35]. Platelets also promote the establishment and growth of tumors. Thus, thrombosis has a significant impact on the morbidity and mortality in cancer.

Oral Contraceptives

Hormonal therapy increases the risk of VTE from two- to fourfold compared with control women. First- and third-generation oral contraceptives confer a higher risk than the second-generation oral contraceptives. Oral contraceptives with ≤35 μg of ethinyl estradiol carry a lower risk of venous thrombosis than those with 50 μg. So far, no single mechanism has been identified to explain the thrombogenicity associated with these medications. Presently, it is believed to be a combination of the direct effect of estrogens on the vascular wall, alterations in coagulation factors, and endothelial dysfunction along with other risk factors unique to the patient.

Pregnancy

Normal pregnancy is associated with a shift of coagulation and fibrinolytic systems toward hypercoagulability. Although these changes have likely evolved to minimize the risk of peripartum blood loss, they also increase the risk of thrombosis. One in 2000 women develops venous thrombosis during pregnancy, and PE is a leading cause of maternal mortality after delivery. The greatest risk of a thrombotic event occurs during the postpartum period. The risk of VTE persists for at least 12 weeks postpartum and is fivefold higher than the risk in pregnancy. However, the absolute risk of developing clinically important VTE during pregnancy or postpartum is low clearly less than 1%.

Arterial Thrombosis

Overview

Arterial thrombosis ranges from large vessel occlusions that result in myocardial infarction, stroke, peripheral arterial vessel occlusion, and ischemic bowel syndrome to small vessel occlusions that result in digital ischemia, acral cyanosis, and vasculitis. Of these, ischemic heart disease and stroke are leading causes of mortality globally, causing 12.9 million deaths in the year 2010 (one in four deaths worldwide) [5]. Pathologic arterial thrombosis is characterized by a complex interaction between acquired environmental conditions and inherited genetic factors.

Etiology

1. Arterial thrombosis is most often associated with ongoing cardiovascular disease as seen with diabetes mellitus, hyperlipidemias, hypercholesterolemia, or vasculitis due to connective tissue disorders or antiphospholipid antibody syndrome.
2. Hematologic conditions associated with arterial thrombosis include sickle cell anemia, heparin-induced thrombocytopenia and thrombosis syndromes (HITTS), thrombotic thrombocytopenic purpura (TTP), hemolytic uremic syndrome (HUS), purpura fulminans due to homozygous protein C or S deficiency, paroxysmal nocturnal hemoglobinuria (PNH), and myeloproliferative disorders (described below in special situations).

Pathogenesis

Arterial thrombi form under high blood flow conditions and are primarily made of platelet aggregates (“white clots”). The inciting event is disruption of an atherosclerotic plaque that releases thrombogenic material (tissue factor, lipid droplets, von Willebrand factor, collagen, thrombospondin, fibronectin, etc.) into the arterial circulation and results in platelet adhesion and aggregation. Platelet receptors GPVI and GPIb-IX have an important role in platelet-collagen and platelet-vWF interactions, respectively [36]. These initial events are followed by activation of the coagulation cascade and fibrin production, which stabilizes the platelet plug.

Risk Factors

Elevated Homocysteine

Elevated plasma homocysteine is a risk factor for both arterial and venous thrombosis. Hyperhomocysteinemia can be caused by inherited and acquired etiologies.

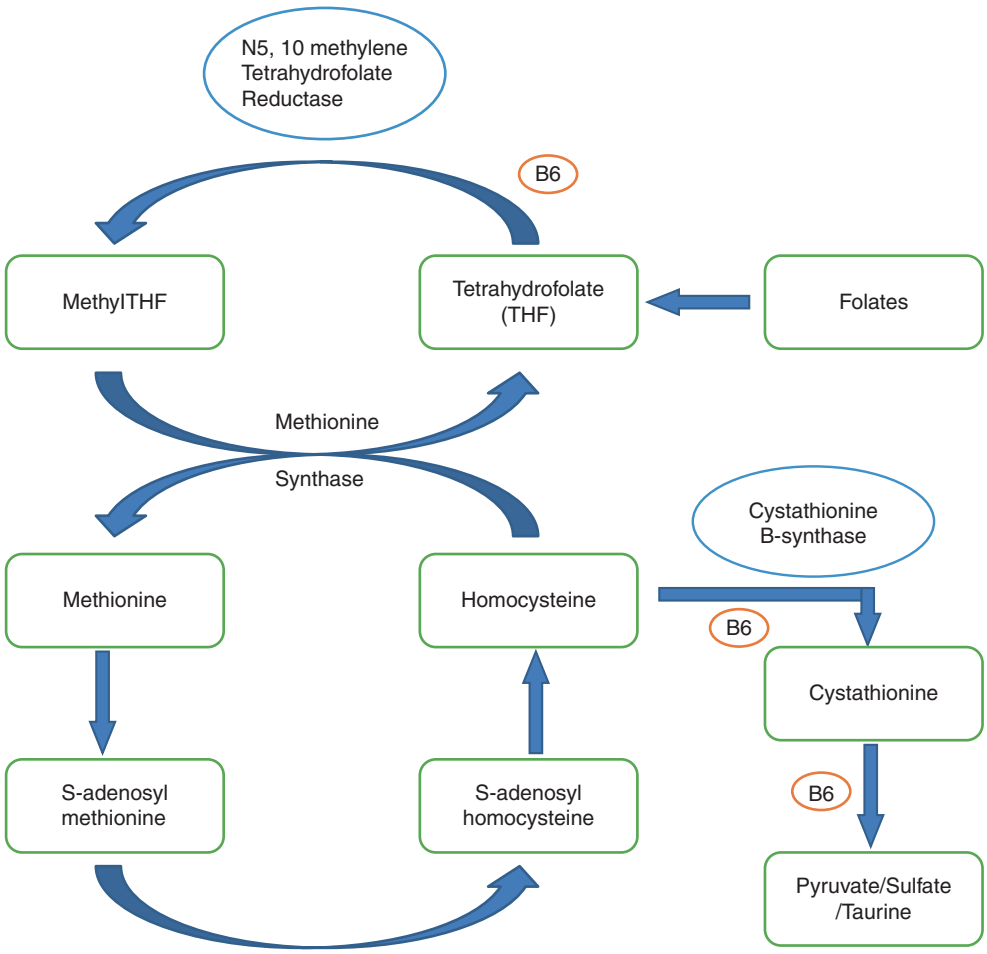

Fig. 16.2 Homocysteine metabolism. Homocysteine is metabolized by two enzymes, methionine synthase and cystathionine β-synthase. N5,N10-methylene-tetrahydrofolate reductase makes an essential cofactor for methionine synthesis. B12 is a cofactor for methionine synthase, and B6 is a cofactor for cystathionine β-synthase

(a) Etiology of elevated homocysteine levels: Homocysteine is an intermediary amino acid that is formed by the conversion of methionine to cysteine (Fig. 16.2). Homocysteine is converted to cysteine by the enzyme cystathionine-β- synthase (CBS), in the presence of vitamin B6 as a cofactor. Re-methylation of homocysteine produces methionine, and this step is catalyzed by methionine synthase (MS) in the presence of methylcobalamin. Methylene tetrahydrofolate reductase (MTHFR) with its cofactor, folate, has a role in re-methylation by producing the 5-methyl THF, which supplies the methyl group.

Congenital: Severe hyperhomocysteinemia with hypercystinuria rarely occurs due to homozygous deficiency of CBS. It is characterized by VTE and premature atherosclerosis in addition to ocular and developmental abnormalities and mental retardation.
Acquired: Mild elevations in homocysteine levels are common, seen in 5–7% of the population. It is usually elevated in end-stage renal disease. The most common cause of mild hyperhomocysteinemia is a variant of MTHFR caused by a cytosine to thymine substitution at nucleotide 677 (C677T). This does not result in thrombophilia. Vitamin deficiencies (B6, B12, or folate) also result in mild to moderate elevations. Hyperhomocysteinemia is characteristic of VTE observed in patients with vitamin B12 deficiency. Modest increases in plasma homocysteine levels are needed for a prothrombotic effect. While many studies suggest increased atherosclerosis or vascular disease with vitamin deficiencies, the data are inconsistent [37, 38].

(b) Elevation of plasma homocysteine results in endothelial cell dysfunction (Fig. 16.3): The normal anticoagulant surface of endothelium is converted to a prothrombotic surface manifesting by reduced protein C activation as result of reduced thrombomodulin (a receptor protein for protein C activation by thrombin on endothelium) expression, reduction in tissue plasminogen activator activation of plasminogen, increased tissue factor expression, reduced nitric oxide production, increased factor V expression, and enhanced LP(a) binding to fibrin. Elevation of plasma homocysteine also results in smooth muscle cell proliferation, lipid peroxidation, and oxidation of LDL, all factors that contribute to atherogenesis and arterial thrombosis. Patients with elevated homocysteine should be treated with folate.

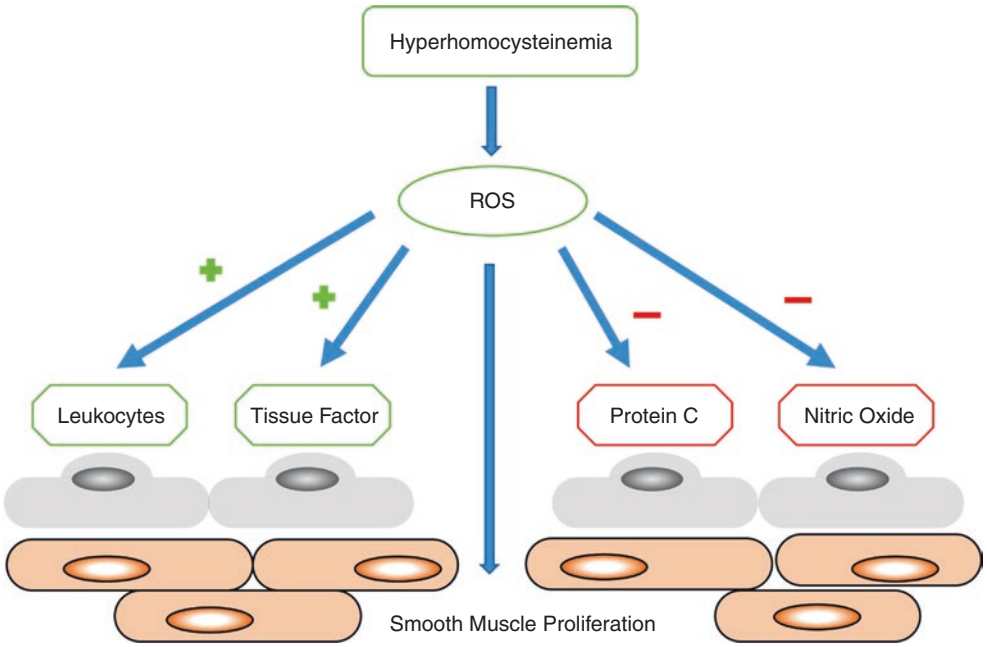

Fig. 16.3 The relationship of homocysteine and endothelial dysfunction. In hyperhomocysteinemic states, there is increased reactive oxygen species (ROS). ROS is injurious to the anticoagulant function of endothelium. It inactivates thrombomodulin, a receptor for protein C activation by thrombin, producing reduced protein C activation and loss of a constitutive anticoagulant. Also ROS uncouples endothelial NO synthase allowing for reduced NO production and more ROS. Concomitantly, ROS increases tissue factor mRNA and protein expression in endothelium. It also stimulates leukocyte migration and infiltration and smooth muscle proliferation. The sum of these activities is vascular dysfunction leading to arterial thrombosis

C-Reactive Protein (CRP)

CRP is an important inflammatory marker that also has a pivotal role in atherogenesis. In addition to facilitating LDL uptake by macrophages, it also increases the expression of adhesion molecules and thereby increases atherosclerotic plaque formation.

CRP contributes to regulating platelet function, the extrinsic coagulation cascade and the fibrinolytic system. Since inflammation upregulates CRP expression, this protein provides an important mechanistic link between inflammation and thrombosis. Additionally, activation of platelets and the coagulation system regulates CRP structure and function suggestive of the bidirectional cross talk between inflammation and coagulation.

Lipoprotein (a)

Lp (a), described in detail above in venous thrombosis, is atherogenic, targeting LDL away from its clearance receptor and prothrombotic by interfering with constitutive plasmin-induced fibrinolysis.

Special Situations: Combined Venous and Arterial Thrombosis

Although the arterial and venous systems have obvious anatomical and structural differences and thrombotic disorders in these systems are considered to have separate pathophysiologies, there is a growing body of data suggesting that this division is oversimplified. There are numerous thrombotic disorders that present with both arterial and venous thrombosis. This overlap provides support to the hypothesis that arterial and venous thrombosis is a continuum that includes vessel wall inflammation, thrombosis, and atherosclerosis [7].

Antiphospholipid Syndrome

Annexin A5 forms a protective shield around vessel membrane surfaces with procoagulant properties. Antiphospholipid antibodies disrupt this shield, exposing the procoagulant phosphatidylserine, thereby predisposing to thrombi in both arterial and venous circulation, spontaneous abortions, preeclampsia, and eclampsia. Increased oxidative stress, tissue factor, factor XI, and complement activation also increase the prothrombotic risk in antiphospholipid syndrome.

Diagnosis of antiphospholipid syndrome (APS) is recognized using the revised Sapporo's criteria. APS is present in patients who meet at least one of the clinical criteria (vascular thrombosis or pregnancy morbidity) and have at least one or more of the antiphospholipid antibodies (IgG/IgM anticardiolipin antibodies, anti-beta-2-glycoprotein, and/or lupus anticoagulant activity) on two or more occasions at least 12 weeks apart [39]. Recent data since the

Sapporo consensus characterization was formulated also show that IgA anticardiolipin and anti-beta-2-glycoprotein antibodies observed over a minimal 3-month hiatus also fulfill criteria.

Paroxysmal Nocturnal Hemoglobinuria (PNH)

PNH is a rare clonal hematopoietic stem cell disorder that is characterized by bone marrow failure, hemolysis, and thrombosis. It results from a somatic mutation in *PIGA* that is required for glycosylphosphatidylinositol (GPI) anchor synthesis. GPI anchor deficiency results in the deficiency of complement inhibitory proteins CD55 and CD59 on the RBC surface that results in chronic complement-mediated hemolysis.

Thrombosis in PNH is multifactorial. Deficiency of CD55 and CD59 leads to prothrombotic microparticles. Chronic hemolysis (causing nitric oxide deficiency due to hemoglobin binding NO), complement activation, and defective fibrinolysis (due to absence of GPI-linked proteins like urokinase-type plasminogen activator receptor) also contribute to the prothrombotic state in PNH. Venous thrombosis (especially cerebral and intra-abdominal) is common than arterial thrombotic events in about 30–40% of PNH patients.

Diagnosis of PNH in the appropriate clinical setting (hypoproliferative anemia/pancytopenia, iron deficiency, atypical thrombosis) with Coombs-negative hemolytic anemia is established by flow cytometry using fluorescent-labeled monoclonal antibodies that bind the GPI-linked proteins, CD55 and CD59.

Thrombotic Microangiopathies (TMAs)

The TMAs include a diverse set of disorders, namely, thrombotic thrombocytopenic purpura (TTP), hemolytic uremic syndrome (HUS), drug-mediated TMAs, and atypical HUS (aHUS) (see Chap. 15). They may be hereditary or acquired and are characterized by arteriolar and capillary thrombosis leading to microangiopathic hemolytic anemia, organ injury, and thrombocytopenia. The mechanisms vary among the disorders (see Chap. 15), but the clinical readout is similar with both arterial and venous microvascular thrombosis.

Heparin-Induced Thrombocytopenia and Thrombosis (HITT)

HITT is an immune complication of heparin use, caused by antibodies directed to complexes containing heparin and a platelet protein, platelet factor 4 (PF4) (see Chap. 15). The anti-PF4/ heparin antibodies bind to and activate platelets resulting in thrombocytopenia and a profound hypercoagulable state. The activated platelets become a platform for thrombin generation resulting in anticoagulant consumption (e.g., protein C) and an accelerated prothrombotic state in the presence of thrombocytopenia. It affects 0.2–3% of patients on heparin therapy.

Immunothrombosis

Immunothrombosis is a newly identified innate immune response that recognizes external pathogens (e.g., bacteria) and inhibits their dissemination and survival by formation of thrombi inside blood vessels [40]. This pathogen defense mechanism is triggered by local accumulation of innate immune cells (neutrophils and monocytes). Under physiologic conditions, it operates without causing the host any damage, but if uncontrolled it can result in thrombotic events.

Activated monocytes potentiate thrombosis by releasing microparticles containing tissue factor. Activated neutrophils release neutrophil extracellular traps (NETs) that are composed of a matrix of DNA and histones (citrullinated histones). NETs have antibacterial and procoagulant properties. NETS are a charged surface for factor XII autoactivation. They also bind vWF, activate platelets, and inactivate anticoagulants (tissue factor pathway inhibitor/TFPI and thrombomodulin) by neutrophil elastases and myeloperoxidase that populate their surfaces. The process of extrusion of granular enzymes and de-condensed nuclear material (chromatin and histones) from an activated neutrophil is now widely referred to as NETosis (Fig. 16.4).

Myeloproliferative Neoplasms (MPNs)

Myeloproliferative neoplasms are a group of clonal stem cell disorders that are associated with a high thrombotic risk (arterial and venous). The pathogenesis of thrombosis in MPNs is complex. The MPN clone-derived blood cells (red cells, white blood cells, and platelets) have prothrombotic features like procoagulant and proteolytic properties. Prothrombotic and proadhesive changes also take place in the endothelium leading to increased attachment of blood cells. The JAK2 V617F polymorphism in Janus kinase 2 gene is associated with 90% of polycythemia vera patients, and 50% of essential thrombocytosis is a gain-in-function change that populates vessel wall in addition to bone marrow myeloid cells. A vasculopathy associated with JAK2 has been recognized. Additionally, elevated white blood cells have been shown to be a risk factor for thrombosis in some mouse models of MPN. Increased NETosis has come to light as a possible culprit mechanism.

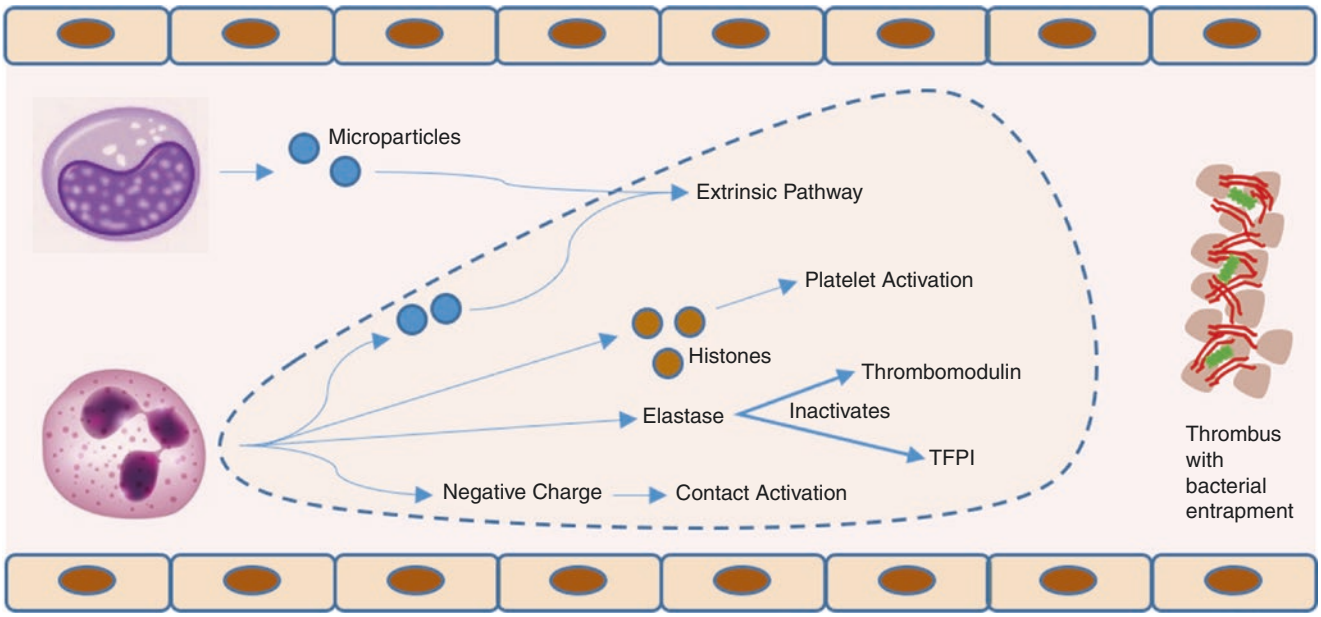

Fig. 16.4 Basic principles of immunothrombosis. Neutrophil extracellular trap (NET) is released from the neutrophil (cell on bottom left). It is a matrix of DNA and histones (citrullinated histones) (brown circles – H3, H4). Their surface is negatively charged that activates coagulation by means of contact activation (factor XII autoactivation). Circulating microparticles (blue circles) from monocytes (cell on top left) and other cells contain tissue factor and activate the extrinsic pathway of coagulation. Histones activate platelets. Neutrophil elastase is released and inactivates thrombomodulin (has anticoagulant activity) and TFPI (tissue factor plasminogen inhibitor). This array of activities results in thrombus formation (platelets cross-linked by fibrin to the right of the figure) with bacterial entrapment (green rectangles), thereby preventing dissemination of infectious organisms

Molecular Regulators of Arterial and Venous Thrombosis

Inflammation and thrombosis are closely linked. One can argue that hemostasis and thrombosis are a subset of the inflammatory system. Factors that regulate inflammation also influence hemostasis and thrombosis. An essential function of the vessel wall is maintaining blood fluidity by inhibiting the onset of coagulation and platelet aggregation and promoting fibrinolysis. When subjected to a pro-inflammatory stimulus, vessel wall and its endothelium lose their antithrombotic properties allowing for the generation of a prothrombotic milieu. Hence, understanding the molecular mechanisms that maintain normal endothelial and vascular smooth muscle function is critical to the development of novel therapeutic strategies for thrombotic disorders. Recent studies identify two transcription factors, KLF2 and KLF4, as nodal regulators of the essential functions of the endothelium that include maintenance of blood in the fluid state [42]. It is interesting to note that while the presence of any one of these factors is sufficient for survival, the absence of both factors is incompatible with life and is associated with acute death due to profound deregulation of the coagulation system, myocardial infarction, heart failure, and stroke. As would be expected, both the KLFs directly regulate the expression of key endothelial genes that are essential for modulation of hemostasis and thrombosis, e.g., thrombomodulin (TM), plasminogen activator inhibitor-1 (PAI-1), and tissue factor (TF).

Oscillatory or turbulent non-unidirectional shear stress activates pro-inflammatory and procoagulant transcription factors, activation protein-1 (AP-1) and nuclear factor kappa B (NFκB), thus inducing a pro-inflammatory and prothrombotic surface. In contrast, steady or pulsatile, unidirectional laminar shear stress induces anti-inflammatory and anticoagulant genes. The key shear transcription factors that determine the expression of these anti-inflammatory and anticoagulant genes are Kruppel-like factor 2 and 4 (KLF2 and 4) and nuclear factor erythroid 2-like 2 (NFE2L2 or commonly referred to as Nrf2). While KLF2 and Nrf2 collectively regulate approximately 70% of the shear stress-elicited genes, Nrf2 potently induces anti-inflammatory and antioxidant enzymes, while KLF2 induces anti-inflammatory and anticoagulant proteins, specifically endothelial nitric oxide synthase (eNOS) and thrombomodulin (TM). Further, KLF2 inhibits pro-inflammatory transcription factors, AP-1 and NFκB, thus providing an additional effect on pro-inflammatory and fibrinolytic pathways [41]. Thus, the response by the endothelium to shear stress is crucial to normal vascular function that includes maintenance of an anti-inflammatory, antithrombotic, anticoagulant, and profibrinolytic state. Upregulation of endothelial KLF2 and KLF4 under conditions of laminar

shear stress induces the expression of anti-inflammatory and antithrombotic proteins, including TM. Conversely, loss of KLF2 or KLF4 leads to enhanced expression of tissue factor and adhesion receptors, e.g., vascular cell adhesion molecule 1 (VCAM-1), and is associated with a prothrombotic phenotype. Finally, dual endothelial deficiency of KLF2 and KLF4 leads to a loss of an intact vasculature, altered blood fluidity, and death – observations that speak powerfully to the central importance of these factors in vascular biology [42].

While free radicals, reactive oxygen/nitrogen species (ROS/RNS), hydrogen peroxide, and hydrogen sulfide have important roles in intra- and intercellular signaling, their production and quenching is a tightly regulated process to prevent cellular damage. Thus, failure to counteract excessive ROS production and modulation of the antioxidant defense system in the endothelium results in endothelial dysfunction characterized by diminished bioavailability of nitric oxide, a key risk factor for cardiovascular disease. Nrf2 is identified as a major regulator of the oxidant/antioxidant balance [43]. Thus, endothelial Nrf2 is activated by increased ROS production, and upregulation of Nrf2 prevents cellular activation of endothelial cells. This highlights the importance of Nrf2 in preserving endothelial function in the presence of oxidative stress. So far, the importance of these genes in the regulation of vascular homeostasis has been primarily in cellular and animal models. Confirmation of the importance of these observations at the bedside are under investigation.

Summary

Arterial and venous thromboses are a significant cause of morbidity and mortality. Understanding their underlying pathophysiology is therefore of utmost importance. Both these processes are complex and dynamic, often involving a multitude of genetic and acquired predisposing factors. The pathogenesis of arterial and venous thrombosis has traditionally been thought to be distinct, eventually culminating in coagulation system activation. However, recent data suggests that arterial and venous thrombosis may in fact be along a continuous spectrum of the same disease.

Prior to this past decade, platelets, vessel wall, and coagulation factors were the most studied culprits of clot formation. Novel concepts with expanding evidence include the role of the immune system (neutrophils and monocytes) and transcription factors in thrombosis. The term "immunothrombosis" describes the formation of thrombi due to over activation of the innate immune system that may occur in an effort to curb infection. This includes but is not limited to the role of neutrophil extracellular traps or NETs. Finally, alterations in the expression of transcription factors such as KLF2 and KLF4 have been found to be key in regulating the anti-inflammatory and anticoagulant functions of the endothelium. The result of these advances in the understanding of the pathobiology of thrombosis should offer potential opportunities for the development of novel, more efficacious, and safer therapeutic strategies that target thrombosis without altering hemostasis.

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Anticoagulants and Treatment of Venous Thromboembolism

17

Allison Burnett and Jack Ansell

Parenteral Anticoagulants

Indirect Thrombin and/or Xa Inhibitors

Overview

Indirect parenteral anticoagulants include unfractionated heparin (UFH), low-molecular-weight heparin (LMWH), and fondaparinux. Each of these agents requires binding to endogenous antithrombin (AT) via a unique pentasaccharide sequence to catalyze anticoagulation and inhibit the formation of thrombin. UFH was discovered in canine liver cells around 1916. Commercial preparations are now derived primarily from porcine intestine. LMWHs, which became commercially available in the mid-1980s, are created through controlled depolymerization of UFH molecules and exhibit a higher degree of target specificity within the coagulation cascade. Fondaparinux, first available in the 1990s, is a synthetic analog of the unique pentasaccharide sequence that binds to AT and is designed to specifically inhibit a single target within the clotting pathway (Fig. 17.1). Each of these rapid-acting agents has been shown to be effective in preventing and treating arterial and venous thromboembolism.

Key Concepts

- The indirect parenteral anticoagulants inhibit the formation of thrombin (factor IIa) by binding to and catalyzing endogenous AT via a unique pentasaccharide sequence.
- UFH, with its non-specific binding and nonlinear kinetics, requires frequent monitoring via the activated partial thromboplastin time (aPTT) or the anti-factor Xa assay, using evidence-based, standardized dosing and titration protocols.
- LMWHs and fondaparinux have a more predictable pharmacokinetic profile and may be administered in fixed doses without the need for routine monitoring of anticoagulant activity.

Unfractionated Heparin

Pharmacology

Mechanism of Action

Unfractionated heparin (UFH) is a heterogeneous mixture of naturally occurring, highly sulfated carbohydrate chains stored in secretory granules within mast cells. The molecular weight of UFH polysaccharide chains ranges from 3000 to 30,000 Da (mean 15,000 Da). UFH requires binding with antithrombin (AT), an endogenous anticoagulant produced by the liver, to exert its effect. Binding occurs via a unique pentasaccharide sequence found on only one-third of heparin molecules and induces a conformational change within AT, accelerating enzymatic inhibition of several clotting factors, with factors IIa (thrombin) and Xa being most sensitive to this inhibition and most critical in thrombus formation (Figs. 17.1 and 17.2). UFH inhibits these two factors equally in a 1:1 ratio. FXa inhibition requires only binding of UFH, regardless of chain length, to AT via the pentasaccharide sequence, whereas thrombin inhibition requires higher molecular weight UFH chains (≥5400 Da) for formation of a ternary complex between UFH, AT, and thrombin. Once UFH has catalyzed AT, it disassociates and can catalyze additional AT molecules. UFH only binds free-floating thrombin and does not possess fibrinolytic activity. Thus, it will not lyse existing thrombi but does prevent clot propagation and growth [1–3].

Pharmacokinetics/Pharmacodynamics

UFH is poorly absorbed from the gastrointestinal (GI) tract and must be administered parenterally, with intravenous (IV) infusion or subcutaneous (SC) injection being the preferred

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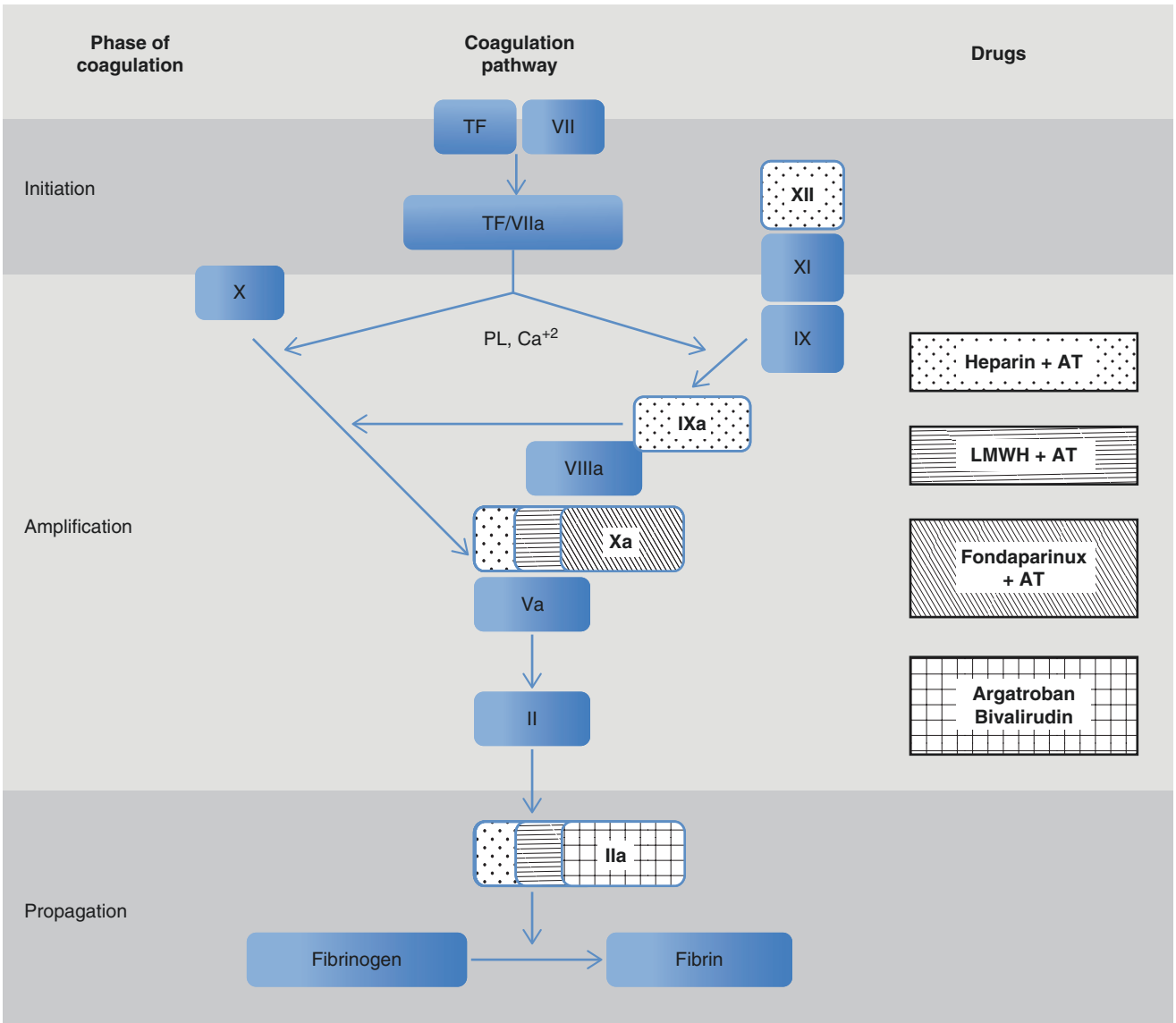

Fig. 17.1 Site of action of parenteral anticoagulants within the coagulation cascade. *AT* anthithrombin, *Ca* calcium, *LMWH* low-molecular-weight heparin, *PL* phospholipid, *TF* tissue factor

routes of administration [1]. The IV route is most commonly employed for therapeutic purposes. An IV bolus followed by a continuous infusion provides an immediate anticoagulant effect and rapid attainment of therapeutic plasma concentrations [4]. When administered SC, bioavailability is reduced to 30–70%, depending on the dose, and the onset of anticoagulation is delayed by 1–2 h. The SC route is most commonly used for venous thromboembolism (VTE) prevention. If the SC route is chosen for treatment of VTE, the dose should be ~10–20% higher than the therapeutic IV dose to overcome the diminished bioavailability [1–3].

UFH binds extensively to the endothelium, macrophages, and plasma proteins other than its intended target, AT, which reduces bioavailability. Plasma levels of heparin-binding proteins vary dramatically over time, particularly in acutely ill patients, rendering the anticoagulant response to heparin unpredictable. Due to intra- and interpatient variability in dose response and changes in patient response over time, UFH requires routine monitoring and dose adjustments via standardized protocols [1, 3].

UFH is cleared from systemic circulation via an initial saturable, macrophage-mediated process and a second, slower non-saturable renal-mediated process. As the initial process becomes saturated with prolonged therapy or higher dosing, clearance becomes dependent on the slower non-saturable process, leading to a nonlinear dose response and a variable half-life (Table 17.1) [1, 2].

Clinical Indications

As the only parenteral anticoagulant for several decades, UFH has gained FDA approval for use in several thromboembolic indications [5], including the following:

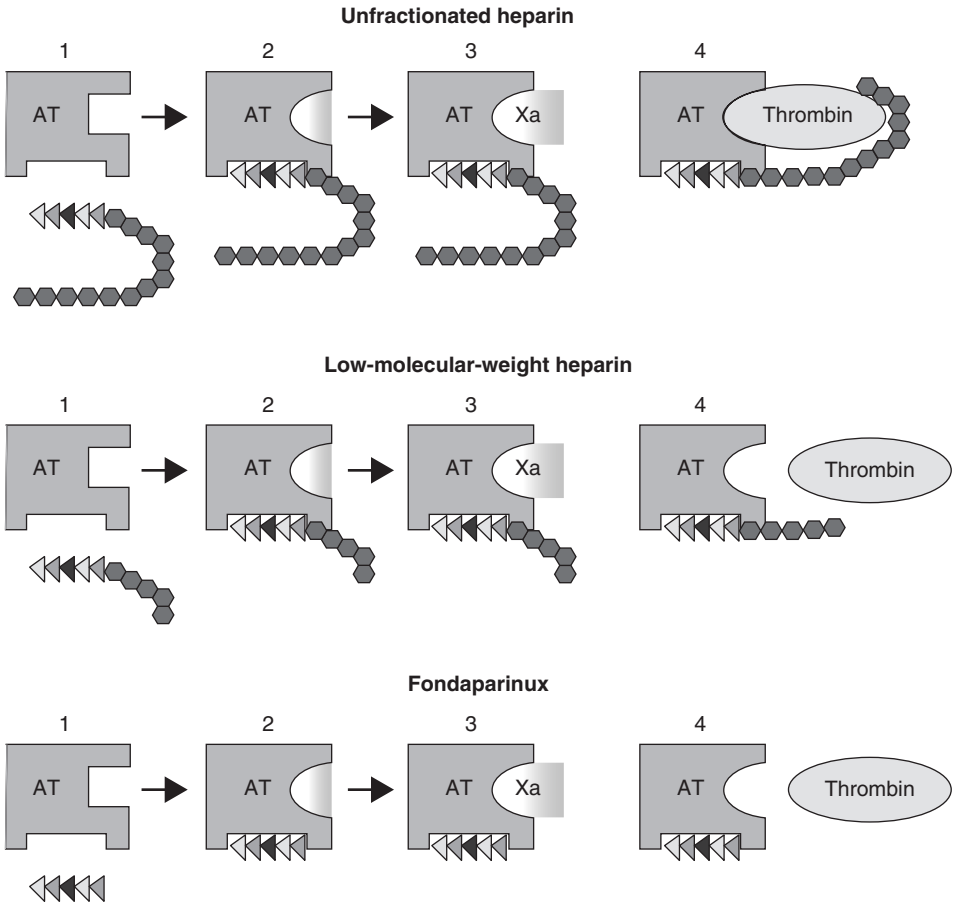

Fig. 17.2 Mechanism of action of indirect parenteral anticoagulants. *AT* antithrombin, *Xa* Factor Xa

- Prevention and treatment of deep venous thrombosis (DVT) and pulmonary embolism (PE), collectively known as venous thromboembolism (VTE)
- Atrial fibrillation and flutter
- Arterial vascular surgery
- Cardiac surgery
- Anticoagulation of device circuits in extracorporeal membrane oxygenation (ECMO) and renal replacement therapies

UFH is generally preferred in patients with severe renal impairment, as it is primarily cleared through non-renal mechanisms and does not require renal dose adjustments. Given its short half-life, it may be preferred in clinical settings that require a rapid offset or reversibility of anticoagulant effect, such as cardiothoracic or vascular surgery [1, 2]. Use of UFH in many settings, such as for treatment of acute VTE, is diminishing with the advent of direct oral anticoagulants (DOACs), which are easier to manage and are safer and more convenient in appropriately selected patients.

Clinical Dosing and Management

Note: dosing of IV UFH for interventional procedures varies widely and is beyond the scope of this chapter. Readers are referred to societal guidelines for detailed procedural dosing information. Dosing information presented here is for common indications encountered by providers caring for patients on medical wards, critical care areas, or emergency departments.

Initiation of Therapy

When used for prevention of VTE, UFH is administered SC at a dose of 5000 units SC BID-TID. While no head-to-head trials of these dosing frequencies have been conducted, indirect evidence suggests TID dosing may be more efficacious at the expense of more bleeds [6]. The short half-life of UFH supports more frequent dosing to avoid prolonged drug-free intervals. Although often done in clinical practice, increasing the dose to 7500 units TID in obese patients may not provide increased efficacy and may cause more bleeds [7].

When administered for treatment, use of a bolus and weight-based dosing (using actual body weight) has been shown to rapidly attain therapeutic levels of anticoagulation [4]. Common dosing for acute VTE includes an 80 unit/kg bolus, followed by an 18 unit/kg/h continuous infusion [4]. Though a less commonly employed strategy, therapeutic UFH administered via weight-based, fixed interval SC dos-

Table 17.1 Parenteral anticoagulants

Drug	Pharmacokinetics	Drug interactions	Routine measurement(s)	Reversal	Dosing	Dosage adjustments
Unfractionated heparin	Half-life (h): ~1.5	Concomitant antihrombotics	IV: lab-specific aPTT equivalent to an anti-factor Xa level of 0.3–0.7 units/ml[a]	Protamine 1 mg for each 100 units of UFH administered within the last 2–3 h Alternative: Protamine 50 mg IV over 10 min	*Prophylaxis:* 5000 units SC BID-TID	Use standardized, weight-based dosing protocol to maintain therapeutic aPTT or anti-factor Xa
	Peak effect (h):				*VTE treatment:* 80 units/kg bolus followed by continuous infusion of 18 units/kg/h or 333 U/kg SC first dose then 250 units/kg SC q12h[b, c] *Acute coronary syndromes:* 60 units/kg bolus followed by continuous infusion of 12 units/kg/h	
	IV: immediate		SC: consider aPTT 6 h after injection on the third day to confirm therapeutic level			
	SC: 1–2 h					
	Renal clearance: minimal		Platelet count q 3 days for first for first few weeks to monitor for HIT			
LMWHs Enoxaparin Dalteparin	Half-life (h)[e]: 3–7	Concomitant antithrombotics	Routine measurement of anticoagulant activity not needed	Protamine 1 mg for each 1 mg or 100 anti-Xa units of LMWH given in previous 8 h Alternative: Protamine 50 mg IV over 10 min	VTE prophylaxis: 40 mg SC once daily (moderate risk) 30 mg SC BID (high risk)	Estimated CrCl 15–29 ml/min: VTE prophylaxis 30 mg SC once daily Treatment dosing 1 mg/kg SC q 24 h Estimated CrCl <15 ml/min: Avoid use
	Peak effect (h): 3–5					
	Renal clearance: >50%		May consider anti-factor Xa level if:		Treatment dosing: 1 mg/kg SC q12h or 1.5 mg/kg SC q24 h[b, c, d]	
			Changing/impaired renal function			
			Extremes of weight			
			Pregnant			
			Platelet count q3 days for first few weeks	Only provides ~60% reversal of LMWH activity	VTE prophylaxis: 2500 units SC once daily (moderate risk) 5000 units SC once daily (high risk)	Estimated CrCl <30 ml/min: No specific dose adjustments provided
			Serum creatinine			
					Treatment dosing: 200 units/kg SC q 24 h or 100–120 units/kg SC q12h[b, c, d]	

Fondaparinux	Half-life(h)[e]: 17–21	Concomitant antithrombotics	Routine measurement of anticoagulant activity not needed	None currently May consider rFVIIa	VTE prophylaxis: 2.5 mg SC once daily	Estimated CrCl 30–50 ml/min: Use with caution
	Peak effect (h): 2–3		Serum creatinine		Treatment dosing: [b, c] 5 mg (<50 kg) 7.5 mg (50–100 kg) 10 mg (>100 kg)	Estimated CrCl <30 ml/min: Avoid use
	Renal clearance: >80%					
Parenteral DTIs						
Argatroban	Half-life: ~40–50 min[f]	Concomitant antithrombotics	aPTT 1.5–3× mean normal	No specific antidote	HIT: 0.5–2 mcg/kg/min, depending on clinical status and hepatic function	
	Peak effect (h): immediate					
	Clearance: primarily hepatic					
Bivalirudin	Half-life[e]: ~25 min		aPTT 1.5–2.5× mean normal	Cessation of infusion is likely sufficient given extremely short half-life	HIT: 0.05–0.15 mg/kg/h, depending on renal function	Use standardized dosing protocol to maintain therapeutic aPTT
	Peak effect (h): immediate					
	Clearance: primarily plasma esterases, minimal renal					

Abbreviations: aPTT activated partial thromboplastin time, *BID* twice daily, *HIT* heparin-induced thrombocytopenia, *IV* intravenous, *LMWH* low-molecular-weight heparin, *SC* subcutaneous, *TID* three times daily, *UFH* unfractionated heparin, *VTE* venous thromboembolism

[a]Therapy target listed is for acute VTE. Therapy target for cardiac indications is approximately 10% lower than for VTE

[b]If a conventional approach with warfarin is used, it should be started as soon as feasible and *overlapped* with parenteral anticoagulant for minimum of 5 days and until INR >2

[c]When used with dabigatran or edoxaban for VTE, a minimum of 5 days parenteral anticoagulant monotherapy; then *switch* to dabigatran or edoxaban immediately once IV UFH infusion stopped or at the time the next dose of LMWH/fondaparinux would be due. *Do not* overlap IV UFH and dabigatran or edoxaban

[d]May be used as monotherapy in VTE patients with active malignancy or pregnancy

[e]In normal renal function

[f]Normal hepatic function

ing at 250 units/kg SC every 12 h has been shown to be effective for acute VTE treatment, as well [8, 9]. In cardiac indications, such as acute coronary syndrome (ACS), dosing is typically lower, with a 60 unit/kg bolus and a 12 unit/kg/h continuous infusion [1]. Bolus dosing is essential to saturate all the intravascular glycosaminoglycan binding sites for heparin to rapidly achieve therapeutic levels but may be deleted if bleeding risk is perceived to be very high (Table 17.1).

Maintenance Dosing and Titration

UFH therapy, but not prophylaxis, requires routine monitoring of anticoagulant activity [1, 3]. UFH may be monitored with the activated partial thromboplastin time (aPTT) or the anti-factor Xa assay. Neither has been rigorously evaluated in clinical trials nor been shown to be superior to the other [1]. The aPTT is not standardized and will vary among laboratory reagents and instrumentation. It is also impacted by variability in plasma proteins and circulating clotting factors [1, 3]. An aPTT range of 1.5–2.5× the mean control aPTT value for the laboratory has traditionally been used [10]. However, variations in reagents and instrumentation across labs require each institution to establish their own therapeutic aPTT range and mean control aPTT for the laboratory. This range should correlate with a plasma heparin concentration of 0.3–0.7 IU/ml by anti-factor Xa assay [1]. Conversely, the anti-factor Xa assay itself may be used to monitor UFH therapy. This assay, which does not depend on thromboplastin reagents, is insensitive to plasma proteins, and may improve monitoring outcomes, has gained in popularity as it has become cost equivalent to the aPTT and is more readily available than in prior years [11]. As with the aPTT, anti-factor Xa assays have yet to be standardized, and results can vary between laboratories. The aPTT and anti-Xa levels are rarely concordant, and routine monitoring of both is discouraged [12]. In patients with heparin resistance (requiring >35,000 units UFH/day to achieve therapeutic aPTT) or with elevated baseline aPTT due to antiphospholipid antibodies, the anti-Factor Xa assay may provide more accurate monitoring of heparin [1] (Table 17.1).

Frequency of Monitoring

Use of the aPTT requires assessment of a baseline value, as it may be elevated even in the absence of anticoagulation, whereas the anti-Xa will be normal (zero) in the absence of anticoagulation. An aPTT or anti-Xa level should be obtained 6 h after initiating an UFH infusion to allow achievement of steady state. Levels should subsequently be measured every 6 h (especially after each dose change) and adjusted per an institution-specific protocol until two sequential therapeutic levels are achieved. Then, monitoring may be decreased to once daily [1, 3] (Table 17.1).

All patients on UFH should have a platelet count performed at least every 3 days for the first 2 weeks of therapy, and periodically thereafter, to monitor for heparin-induced thrombocytopenia (HIT) that has a 1–5% incidence [1, 13].

Managing Invasive Procedures

For procedures requiring minimal to no residual anticoagulant effect, IV UFH should be discontinued 4–6 h prior to the procedure, and twice daily SQ therapeutic UFH should be held for approximately 12 h prior [14]. Interruption of prophylactic UFH varies by procedure and should be discussed with the surgical provider or team. Resumption post-procedurally should be based on achievement of hemostasis, patient's bleeding risk, presence of underlying indication for anticoagulation, and the associated thrombotic risk.

Managing Bleeding and Reversal

Adverse Events

The most common adverse effects of UFH are bleeding and heparin-induced thrombocytopenia (HIT) [1, 2]. Other known complications include osteoporosis and an increase in hepatic enzymes. The risk of bleeding with UFH is associated with the intensity and stability of therapy. Other factors that increase the risk of bleeding with UFH include, but are not limited to, increasing age, renal function, concomitant administration of drugs that affect hemostasis (e.g., antiplatelets, NSAIDS, fibrinolytics), recent surgery, and trauma [1]. In addition to general supportive measures (withdrawal of anticoagulant, hemodynamic monitoring, fluid resuscitation, transfusion of blood products, etc.), patients with serious bleeding may be given protamine sulfate, which binds and neutralizes UFH [15]. Because it is derived from salmon sperm, it should not be used in patients with a fish allergy. Anaphylactoid reactions to protamine sulfate can occur, even in the absence of a fish allergy, and administration by slow IV infusion is recommended to reduce this risk [15]. In clinical practice, for major bleeding associated with UFH, a dose of 50 mg IV protamine may be administered over 10 min, with redosing as needed for refractory bleeding. Because UFH has a short half-life, if more than 4–6 h have passed since the last UFH administration, protamine is unlikely to provide any benefit [15] (Table 17.1). Note that protamine sulfate itself can induce contact activation of factor XII. Overdosing of protamine sulfate can, in fact, induce blood coagulation through contact activation.

Immune-Mediated Heparin-Induced Thrombocytopenia (HIT)

Immune-mediated HIT, a potentially fatal prothrombotic condition, has been reported to occur in up to 5% of patients exposed to UFH. Hallmark signs include a 50% decrease in platelet count from patient's baseline prior to UFH exposure that occurs between days 5 and 15 after exposure. Investigation of HIT should include cessation of any heparin products (UFH, LMWH), calculation of a pretest probability

Table 17.2 4T pretest clinical probability score for heparin-induced thrombocytopenia (HIT)

4Ts category	2 points	1 point	No points
Thrombocytopenia	Platelet count fall >50% and nadir ≥20	Platelet count fall 30–50% or platelet nadir 10-19 K	Platelet count fall <30% or any platelet fall with nadir <10 K
Timing	Clear onset days 5–10 or platelet fall ≤1 day (with prior heparin exposure <30 days)	Unclear onset days 5–10, or >10 days or platelet fall ≤1 day (with prior heparin exposure 30–100 days)	Onset ≤day 4 without previous exposure to heparin
Thrombosis	New confirmed thrombosis or skin necrosis at heparin injection sites or acute systemic reaction following heparin administration	Progressive thrombosis or non-necrotizing skin lesions at heparin injection sites or suspected (unconfirmed) thrombosis	None
O**t**her causes of thrombocytopenia	None	Possible	Definite

Risk strata: 0–3 = low, 4–5 = intermediate, 6–8 = high, $K = 1000$

via the 4T score (Table 17.2), and initiation of an alternative anticoagulant (such as a parental DTI or fondaparinux) in at-risk patients. While associated with significant morbidity and mortality if untreated, accurately selecting patients for HIT testing and treatment via the 4T score, which has a 95% negative predictive value (NPV), is critical, as therapeutically anticoagulating a patient with a true (vs prothrombotic) thrombocytopenia significantly increases their risk of bleeding. For patients with a 4T score of ≤ 3, no antibody testing is indicated. For those with a score >3, additional consideration and possibly antibody testing are warranted [13]. Consultation with an anti-thrombosis expert when HIT is suspected is strongly recommended.

Osteoporosis

UFH therapy has been shown to reduce bone density in up to one-third of patients treated for >1 month, with 2–3% experiencing bone fractures [16]. Alternative agents with a lower incidence of osteoporosis, such as LMWH, are preferred for long-term therapy.

Abnormal Liver Function Tests

UFH, through unknown mechanisms, has been associated with elevations in liver transaminases (AST, ALT) that return to normal upon withdrawal of the drug.

Transitioning Between Anticoagulants

Transitioning to UFH

- When transitioning *to IV/SC UFH* from LMWH, fondaparinux or a DOAC, the IV/SC UFH should be started at the time the next dose of the LMWH, fondaparinux, or DOAC would be due.
- When transitioning *to IV/SC UFH* from warfarin, IV/SC UFH should be started once the INR is <2.

Transitioning from UFH

- Because of UFH's short half-life, when transitioning *from IV UFH* to an alternative rapid-acting anticoagulant (LMWH, fondaparinux, DOAC), the alternative agent may be started at the same time the IV UFH is stopped.
- Because of a longer half-life with SC administration, when transitioning *from SC UFH* to an alternative rapid acting anticoagulant (LMWH, fondaparinux, DOAC), the alternative agent should be started at the time the next dose of SC UFH would be due.
- When transitioning *from IV UFH* to warfarin, the therapies should be overlapped for ≥5 days and until the INR is >2 for at least 24 h.

Low-Molecular-Weight Heparin (LMWH)

Pharmacology

Mechanism of Action

Two LMWHs are currently available in the United States: enoxaparin and dalteparin. The LMWHs are derived via chemical or enzymatic depolymerization of UFH. Like UFH, LMWHs prevent the propagation and growth of formed thrombi but do not break down existing clots. Also like UFH, LMWH are indirect anticoagulants exerting their anticoagulant effect by binding to AT through a unique pentasaccharide sequence (Fig. 17.2). Because of their shorter length and lower molecular weight (4500–5000 Da), LMWHs are unable to bind AT and thrombin simultaneously (Fig. 17.2). The LMWHs thus inhibit factor Xa to a greater degree than thrombin, with an anti-factor Xa-to-IIa activity ratio ranging from 2:1 to 4:1 [1, 2].

Pharmacokinetics/Pharmacodynamics

LMWHs have an improved pharmacokinetic and pharmacodynamic profile compared to UFH. Because they exhibit a lower degree of non-specific binding to plasma proteins, LMWH possesses linear kinetics, a more predictable dose response and more favorable side-effect profile. As a result, these agents can be given in fixed doses without need for routine monitoring of anticoagulation activity. LMWHs are not absorbed from the gastrointestinal tract and must be administered parenterally. Bioavailability of LMWHs fol-

lowing subcutaneous injection is nearly 100%. Peak anti-Xa activity occurs about 3–4 h following administration, and steady state is achieved after 2–3 doses [1, 2].

Enoxaparin and dalteparin are metabolized in the liver by desulfation and/or depolymerization to lower molecular weight substances with minimal biologic activity. LMWHs are predominantly (>50%) cleared via renal elimination. In normal renal function, the elimination half-lives of dalteparin and enoxaparin range from 3 to 7 h after repeat dosing and attainment of steady-state plasma concentrations. In reduced renal function, the half-life will be prolonged, and a dose reduction or avoidance may be required to avoid accumulation and increased risk of bleeding (Table 17.1) [1, 2].

Clinical Indications

Dalteparin [17] is FDA-approved for:

- VTE prophylaxis in patients undergoing abdominal surgery and hip replacement surgery or with acute medical illness
- Extended treatment of cancer-associated VTE
- ACS

Off-label uses of dalteparin [17] include:

- Acute treatment of non-cancer-associated VTE
- Pregnancy-associated VTE
- Bridging therapy for mechanical prosthetic cardiac valve

Enoxaparin [18] is FDA-approved for:

- VTE prophylaxis in patients undergoing hip or knee arthroplasty and abdominal surgery or with acute medical illness
- Acute treatment of non-cancer-associated VTE
- ACS

Off-label uses of enoxaparin [18] include:

- Bridging therapy for mechanical prosthetic cardiac valve
- Pregnancy-associated VTE
- VTE prophylaxis in bariatric, general, gynecologic, or cancer surgery
- VTE prevention and treatment in pediatric patients
- Bridging therapy during temporary interruption of vitamin K antagonists (VKAs), such as warfarin, in patients at high risk for thromboembolism

Clinical Dosing and Management

Dosing

Prophylactic or treatment doses vary depending on the LMWH preparation. Dalteparin dosing is based on anti-Xa units, whereas enoxaparin is dosed in milligrams (mg). One milligram of LMWH is equivalent to 100 anti-Xa units.

For prophylaxis, dalteparin is dosed at 2500 or 5000 units SC once daily, depending on the patient's VTE risk [17]. Enoxaparin prophylactic dosing is 30 mg SC BID for high-risk patients and 40 mg SC once daily for moderate-risk patients [18]. Optimal VTE prophylaxis dosing in obesity is not known, but increasing the dose by 25–30% has been suggested and is reasonable [3, 19]. In low-weight patients (<45 kg women, <57 kg men), enoxaparin has been shown to accumulate which may increase bleed risk and may warrant a dose reduction or avoidance [3, 18].

When used for treatment, dalteparin may be dosed at either 100–120 units/kg twice daily (BID) or 200 units/kg once daily, and enoxaparin may be dosed at either 1 mg/kg BID or 1.5 mg/kg once daily, using actual body weight and depending on indication [1, 3, 17, 18]. Dose capping of LMWH in obesity is not recommended [19]. Whenever possible, once-daily LMWH is recommended for patient convenience, adherence, and cost. However, use of more frequent BID dosing may be preferred in select populations, such as those with extensive thrombus burden, obesity, or active cancer [3]. Also, BID dosing should be preferred in patients with an increased risk of bleeding, such as in the postoperative setting, as this will avoid the higher-peak anti-Xa activity seen with once-daily dosing. The wide therapeutic index of LMWHs allows for dose rounding, preferably to the nearest commercially available syringe size, to simplify administration as well as reduce risk for dosing errors.

Due to differences in molecular weight and charge, dalteparin exhibits less accumulation in renal dysfunction than enoxaparin. No renal dose adjustments are provided for dalteparin, but it is suggested to monitor anti-Xa levels for patients with an estimated creatinine clearance (CrCl) of <30 ml/min by Cockroft-Gault equation. Enoxaparin prophylaxis and treatment should be dose-reduced for patients with an estimated CrCl of <30 ml/min (along with consideration for anti-Xa monitoring for accumulation) and avoided in those with severe renal impairment (estimated CrCl <15 ml/min) [1–3].

Administration

Both dalteparin and enoxaparin come in pre-filled syringes, which increase the feasibility and convenience of outpatient therapy. LMWHs should be administered subcutaneously in the abdomen once or twice daily. If administration in the abdomen is not possible, these agents may be administered in other areas with appreciable amounts of subcutaneous fat, such as the lateral aspect of the upper arm or thighs.

Monitoring

Routine monitoring of LMWHs is not recommended but may be considered in select populations, such as those with

extremes of weight, changing renal function, or altered pharmacokinetics (e.g., pregnancy, burns, pediatrics, etc.) [1–3, 19]. It is important to note optimal anti-Xa ranges for LMWHs have not been established and observed ranges have not been strongly correlated with clinical outcomes. If monitoring is employed, a chromogenic anti-factor Xa assay calibrated to LMWH should be used. When assessing for accumulation, trough anti-Factor-Xa levels, taken just prior to the next dose, may be used with a target of <0.5 IU/ml. Peak levels of LMWH should be drawn 4 h post-dose, with an expected range of 0.2–0.5 IU/ml for prophylaxis, 0.6–1.0 IU/mL with BID treatment dosing, and 1–2 IU/mL with once-daily treatment dosing [1]. The clinical significance of elevated anti-factor Xa levels is unknown, and there are no suggested dose adjustments to achieve a specific anti-factor Xa level.

Managing Invasive Procedures

The last dose of prophylactic LMWH should be administered at least 6 h prior to procedure and up to 12–24 h prior. Postoperatively, prophylactic LMWH should be resumed no sooner than 6 h after the procedure to mitigate bleed risk [14]. Also, postoperative prophylaxis should not be excessively delayed (e.g., >24 h), in the absence of contraindications, as this may lead to postoperative VTE complications.

For patients receiving therapeutic LMWH, the last dose should be given ~24 preoperatively. Resumption postoperatively should occur at ~24 h for low bleed risk procedures and ~48–72 h for higher bleed risk procedures. Use of step-up approach, with prophylactic dosing at 24 h, is reasonable for prevention of post-op DVT and minimization of bleeding via avoidance of therapeutic anticoagulation in the immediacy [14, 20].

Managing Bleeding and Reversal

Adverse Effects

As with any anticoagulant, bleeding is the most common complication of LMWHs. Risk may be reduced via careful consideration of renal function, safe and appropriate timing of doses, and avoidance of concomitant agents that may alter coagulation status, such as antiplatelets [1, 15]. In the event of a significant bleed, general approaches should be employed, and protamine sulfate should be considered. Renal function should be rapidly assessed to determine if significant accumulation may have contributed to the bleeding event and aid in estimation of remaining duration of drug exposure. Due to limited binding to LMWH, protamine only neutralizes about 60% of LMWH anticoagulant activity. A dose of 50 mg IV may be given if the last dose of LMWH was given in the previous 8 h, with a repeat dose if bleeding is not controlled. If >8 h has passed since last LMWH administration, protamine is not likely to provide benefit.

Heparin-Induced Thrombocytopenia

LMWHs have less interaction with the heparin-binding protein platelet factor 4 (PF4) and consequently are associated with an approximate five- to tenfold lower rate of HIT compared to UFH. However, LMWHs do cross-react with heparin antibodies in vitro and should not be used as an alternative anticoagulant in patients with suspected or confirmed HIT. Platelet counts should be monitored every few days during the first 2 weeks of LMWH therapy and periodically thereafter [1, 13].

Transitioning Between Anticoagulants

Transitioning from LMWH

- When switching *from an LMWH* to an alternative anticoagulant with a rapid onset of action, such as UFH, fondaparinux, or a DOAC, the alternative anticoagulant should be started at the time the next dose of LMWH would have been due.
- When transitioning *from an LMWH* to warfarin, such as in acute VTE or HIT, the LMWH should be overlapped with warfarin for a minimum of 5 days and until the INR is >2 for approximately 24 h. This is due to the long half-life (~40 h) and slow onset of effect of warfarin, coupled with the long half-life of pre-existing, circulating thrombin (~60 h).

Transitioning to LMWH

- When transitioning *to an LMWH* from a DOAC, the LMWH should be started at the time the next DOAC dose would have been due.
- When transitioning *to an LMWH* from IV UFH, the LMWH may be started as soon as the IV UFH is stopped.
- When transitioning *to an LMWH* from SC UFH, the LMWH should be started at the time the next SC UFH dose would have been due.
- When transitioning *to an LMWH* from warfarin, the LMWH should be started when the INR is <2.

Fondaparinux

Pharmacology

Mechanism of Action

Fondaparinux is a synthetic analog of the of the unique pentasaccharide sequence found within porcine-derived UFH and LMWH chains and has a molecular weight of 1728 Da. Like UFH and LMWH, fondaparinux is an indirect anticoagulant, requiring binding to AT to exert its effect (Fig. 17.2). This interaction with AT induces a conformational change that catalyzes targeted binding and inhibition of FXa. Fondaparinux is then released and available to catalyze other AT molecules. Due to its short length compared to UFH, fondaparinux is unable to bind and inhibit thrombin [1].

Pharmacokinetics/Pharmacodynamics

Fondaparinux is >95% bound to AT, exhibits linear pharmacokinetics, and has a highly predictable dose response across a wide range of studied doses. This predictability, along with high bioavailability and a long half-life, minimizes inter- and intra-patient variability and allows fondaparinux to be given in fixed, once-daily doses without the need for routine monitoring and reduced potential for adverse effects. Fondaparinux is not absorbed through the gastrointestinal mucosa. SC administration provides rapid and complete absorption of fondaparinux with 100% bioavailability. Peak plasma concentrations are achieved at approximately 2–3 h after subcutaneous administration, and steady state is achieved after 3–4 doses of once-daily fondaparinux (Table 17.1) [1, 3, 19].

Decreased binding to macrophages and endothelial cells increases the plasma half-life of fondaparinux compared to UFH and LMWH. Fondaparinux is heavily dependent on renal elimination, with up to 77% of drug excreted unchanged in the urine. The terminal half-life is 17–21 h in healthy volunteers, and this will be prolonged in acute or chronic kidney injury. Age (>75 years) and low body weight (<50 kg) are associated with reduced clearance of fondaparinux. In patients with multiple factors that may affect fondaparinux clearance, the effect is likely to be cumulative, and use of an alternative anticoagulant may be indicated. After stopping fondaparinux, the anticoagulant effect will persist for up to 4 days and even longer in patients with reduced clearance [1, 3, 19]. As it does not affect pre-existing circulating thrombin, it is theorized that fondaparinux may afford some degree of residual hemostatic function, should it be needed, at a site of injury. As a synthetic agent, fondaparinux may be used in patients with a documented pork allergy or with religious beliefs precluding pork products.

Clinical Indications

Fondaparinux [21] is FDA-approved for VTE prevention in patients undergoing hip, knee, or abdominal surgery. Based on results from the MATISSE DVT and PE trials [22, 23], fondaparinux is also FDA-approved for acute treatment of VTE in conjunction with warfarin. The most recent CHEST 2016 guidelines recommend VTE treatment with a direct oral anticoagulant (DOAC) over conventional approaches of warfarin overlapped with a rapid-acting parenteral agent such as UFH, LMWH, or fondaparinux [24]. When a patient is not a DOAC candidate, the ACCP 2012 guidelines on antithrombotic therapy for VTE recommend fondaparinux (or LMWH) over UFH for initial parenteral therapy for acute VTE [9].

Off-label uses include:

- Treatment of acute superficial venous thrombosis of the leg
- Acute coronary syndromes (ACS)
- VTE prevention in general surgery
- Heparin-induced thrombocytopenia (HIT)
  - Fondaparinux may be preferred in stable HIT patients with normal renal function as it greatly simplifies management compared to other therapies, such as argatroban and bivalirudin.

Clinical Dosing and Management

Dosing

The prophylactic dose of fondaparinux is 2.5 mg SQ once daily. Prophylactic use in patients <50 kg is contraindicated, as orthopedic studies have shown increased risk for bleeding in low-weight patients. For obese patients, the same 2.5 mg prophylactic dose may be used without need for dose adjustment [1, 21, 25].

When used for treatment, fondaparinux is given in fixed doses based on the patient's weight category (Table 17.2). For obese patients, a standard dose of 10 mg SQ once daily may be used without need for upward dose adjustment. Because it is primarily renally eliminated, it is contraindicated in patients with an estimated CrCl of <30 ml/min by Cockroft-Gault equation, and caution is recommended in those with an estimated CrCl of 30–50 ml/min [1, 3].

Administration

Fondaparinux comes in pre-filled syringes of 2.5, 5, 7.5, and 10 mg strengths, which increases convenience and facilitates outpatient therapy. It is administered subcutaneously in the abdomen once daily. As with LMWHs, if administration in the abdomen is not possible, fondaparinux may be administered in other areas with appreciable amounts of subcutaneous fat, such as the lateral aspect of the upper arm.

Monitoring

While not routinely recommended, if measurement of fondaparinux is indicated (e.g., changing renal function, extremes of weight, or age), plasma concentration is most accurately assessed by use of a chromogenic anti-factor Xa activity assay calibrated to fondaparinux. It is important to note optimal anti-Xa ranges for fondaparinux have not been established and observed ranges have not been strongly correlated with clinical outcomes [1, 3].

Managing Invasive Procedures

Because of its long half-life, prophylactic fondaparinux should be held for at least 48–72 h and therapeutic fondaparinux held for at least 72–96 h prior to invasive procedures requiring minimal to no residual anticoagulant effect. Resumption post-procedurally should be based on achievement of hemostasis, patient's bleeding risks, presence of underlying indication for anticoagulation, and the associated thrombotic risk.

Managing Bleeding and Reversal

Adverse Effects

The most common adverse effect of fondaparinux is bleeding. Risk may be mitigated by administering a first post-procedural prophylactic dose at least 6 h after the procedure or even the next morning, which will not compromise efficacy [26]. Clinicians must ensure a patient has adequate renal function prior to and throughout fondaparinux therapy to avoid bleeding complication associated with accumulation. Concomitant use of fondaparinux and drugs that affect coagulation (e.g., antiplatelets, NSAIDs) poses a pharmacodynamic drug interaction that may potentiate bleeding risk and should be avoided whenever possible. If a patient on fondaparinux does experience a bleed, it is important to rapidly evaluate their current renal function to estimate how long the anticoagulant effect of fondaparinux will persist. Also, clinicians should employ assertive fluid resuscitation to promote renal elimination of fondaparinux if possible. Currently there is no specific antidote for fondaparinux (Table 17.1). It cannot be reversed with protamine, possibly due to its charge, sulfate content, molecular size, or a combination of these factors. Fresh-frozen plasma (FFP) or factor concentrates such as prothrombin complex concentrate (PCC) or recombinant factor VIIa (rFVIIa) have been used, but none of these have been adequately studied. Additionally, factor concentrates have been associated with a risk of thrombosis and should be reserved for clinical situations refractory to general approaches to bleeding management.

Transitioning Between Anticoagulants

Transitioning from Fondaparinux

Clinical situations that might involve transitioning from fondaparinux to an alternative anticoagulant include a desire for a shorter-acting anticoagulant (e.g., UFH, LMWH) prior to an invasive procedure or need for longer-term oral anticoagulation therapy.

- When switching *from fondaparinux* to an alternative anticoagulant with a rapid onset of action, such as UFH, LMWH, or a DOAC, the alternative anticoagulant should be started 24 h after the last dose of fondaparinux.
- When transitioning *from fondaparinux* to warfarin, such as in acute VTE or HIT, fondaparinux should be overlapped with warfarin for a minimum of 5 days and until the INR is >2 for approximately 24 h.

Transitioning to Fondaparinux

The most common clinical situation wherein a transition to fondaparinux from another anticoagulant might occur is HIT.

- Patients with suspected or confirmed HIT who have been receiving UFH (either SQ or IV) or LMWH should have fondaparinux initiated as soon as safely possible, regardless of the timing of the previous dose of UFH or LMWH, to minimize the risk for HIT-associated thrombosis.
- This obviously may place patients at risk of over-anticoagulation if UFH or LMWH has been recently administered and underscores the importance of using the 4T score to determine pretest probability of HIT to most accurately identify at-risk patients warranting a change in therapy.
- For patients with suspected or confirmed HIT that have recently been started on warfarin, vitamin K should be administered, and a parenteral DTI should be initiated.

Direct Thrombin Inhibitors (Argatroban and Bivalirudin)

Overview

Hirudin is a naturally occurring direct thrombin inhibitor (DTI) derived from leech saliva that has high affinity for thrombin. It binds to both the active site and the exosite 1 region on thrombin. Although a recombinant form was once approved for clinical use (lepirudin), it was removed from the market due to excessive bleeding. Two synthetic direct thrombin inhibitors, argatroban and bivalirudin, are currently available for clinical use. Unlike indirect anticoagulants that require binding with AT, DTIs bind specifically and directly to thrombin [1, 27, 28] (Fig. 17.1).

Key Concepts

- Parenteral DTIs are administered via continuous infusion and monitored via the aPTT using standardized dosing and titration protocols.
- Parenteral DTIs are not usually first-line anticoagulant therapies but rather are used in clinical situations that require a parenteral anticoagulant but preclude the use of UFH or LMWH, such as HIT.

Pharmacology

Mechanism of Action

Bivalirudin (2180 Da) is a bivalent synthetic peptide DTI that reversibly binds to thrombin at both the active site and exosite 1. Argatroban (508 Da) is a monovalent non-peptide mimetic DTI and reversibly binds thrombin at the active site only (Fig. 17.3) [28].

Pharmacokinetics/Pharmacodynamics

Argatroban and bivalirudin are administered parenterally and thus do not require absorption. Due to their short half-lives, they are administered via continuous infusion. Both agents produce an immediate anticoagulant effect, and steady-state plasma concentrations are achieved within a few

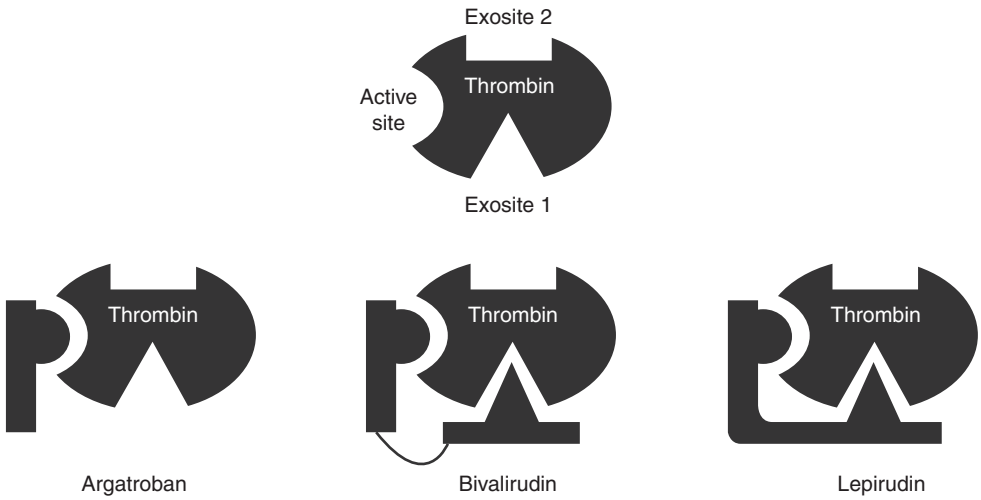

Fig. 17.3 Mechanism of action of parenteral direct thrombin inhibitors (DTIs). The activity of DTIs is produced by direct interaction with the thrombin molecule. Bivalirudin (a bivalent DTI) simultaneously binds exosite 1 and the active site. Argatroban (a univalent DTI) binds only the active site. (Adapted from Baetz et al. [29])

hours of initiation of therapy. The parenteral direct thrombin inhibitors do not bind to plasma proteins or cells and thus produce a more linear and predictable anticoagulant response than unfractionated heparin [1, 27, 28].

Argatroban is metabolized primarily in the liver and excreted in the feces through biliary secretion. Patients with impaired hepatic function have a fourfold decrease in clearance and require dose adjustments. Bivalirudin is metabolized primarily via blood proteases and broken down into the amino acid pool. Approximately 20% of bivalirudin is excreted via the kidneys as unchanged drug. In diminished renal function, the half-life will be prolonged, and dose adjustments are indicated (Table 17.1) [1, 27, 28].

Clinical Indications

FDA-approved indications for argatroban [30] include:

- HIT
- Percutaneous coronary intervention (PCI) with HIT

FDA-approved indications for bivalirudin [31] include:

- Percutaneous coronary interventions with or without HIT

Common off-label use of these two therapies includes circuit patency in cardiopulmonary bypass (CPB) surgeries and extracorporeal membrane oxygenation (ECMO) and renal replacement therapies in patients with a contraindication to UFH, such as HIT.

Heparin-Induced Thrombocytopenia

Due to their mechanism of action, argatroban and bivalirudin have become the mainstays of initial HIT treatment. They do not interact with platelet factor 4 (PF4), and their direct inhibition of thrombin reduces platelet activation and prevents thrombus formation.

Argatroban is FDA-approved and ACCP guideline-recommended for treatment of HIT. Though not FDA-approved, use of bivalirudin for immune-mediated HIT has increased over the last decade, as evidence suggests that target aPTTs are achieved more quickly with a similar or reduced incidence of bleeding as compared to other parenteral DTIs. Additionally, bivalirudin has several practical advantages over argatroban, including less impact on the INR, less reliance on organ elimination, and potentially lower cost. While bivalirudin is not specifically recommended as a treatment option for HIT in the ACCP guidelines, it is noted that "factors such as drug availability, cost, and ability to monitor the anticoagulant effect may influence the choice of agent" [13].

Clinical Dosing and Management

Note: dosing of parenteral DTIs for interventional procedures varies widely and is beyond the scope of this chapter. Readers are referred to societal guidelines for detailed procedural dosing information. Dosing information presented here is for common indications encountered by providers caring for patients on medical wards, critical care areas, or emergency departments.

Initiation of Therapy

Dosing of both argatroban and bivalirudin for HIT is provided in Table 17.1. As discussed previously, both bivalirudin and argatroban may require initial dose adjustments based on their primary method of elimination and individual patient characteristics. Both are administered as a continuous infusion.

Maintenance Dosing and Titration

Use of a standardized, evidence-based protocol for initiation, titration, and maintenance of parenteral DTIs is recommended for HIT treatment. In HIT, argatroban and bivaliru-

din are typically monitored via the aPTT, with a target of 1.5–3× and 1.5–2× the institution-specific control aPTT, respectively. If a patient's baseline aPTT is elevated to within the target range, it may preclude either the use of a DTI or use of the aPTT for monitoring. In these instances, use of an alternative anticoagulant (e.g., fondaparinux) or an alternative assay (e.g., a DTI-specific assay) should be considered. While some institutions have developed DTI-specific assays, they are not commercially available for widespread use. HIT protocols should also incorporate monitoring of other laboratory parameters (e.g., hemoglobin, hematocrit) in order to assess for bleeding [1, 13].

Frequency of Monitoring

The aPTT is typically drawn 2–4 h after start of the DTI infusion (to allow achievement of steady-state plasma concentrations), after any dose adjustments and every 2–4 h until therapeutic. Once the patient has two consecutive therapeutic aPTTs, monitoring may be decreased to once or twice daily.

Bivalirudin mildly affects the INR, with a mean increase of 0.6, whereas argatroban imparts a more pronounced effect [32]. It should be noted this is only a lab artifact and does not convey the same increased bleed risk as an elevated INR with warfarin therapy. It can, however, make transitioning to warfarin very challenging. (See section on transitioning to oral anticoagulation below.)

Managing Invasive Procedures

Based on their short half-lives, parenteral DTIs should be held 2–4 h prior to invasive procedures requiring minimal to residual anticoagulant effect. Note that the half-lives of argatroban and bivalirudin may be prolonged in patients with hepatic and renal impairment, respectively, and require earlier cessation prior to a procedure to allow adequate offset of drug effect. Resumption post-procedurally should be based on achievement of hemostasis, patient's bleeding risks and underlying indication for anticoagulation, and the associated thrombotic risk.

Transitioning Between Anticoagulants

Transitioning to a Parenteral DTI

As with fondaparinux, a common clinical situation wherein a transition to a parenteral DTI from another anticoagulant might occur is HIT.

- Patients with suspected or confirmed HIT who have been receiving UFH (either SQ or IV) or LMWH and cannot be transitioned to fondaparinux (e.g., severe renal impairment) should have a parenteral DTIs initiated as soon as safely possible, regardless of the timing of the previous dose of UFH or LMWH, to minimize the risk for HIT-associated thrombosis.
- This obviously may place patients at risk of over-anticoagulation if UFH or LMWH has been recently administered and underscores the importance of using the 4T score to determine pretest probability of HIT to most accurately identify at-risk patients warranting a change in therapy (Table 17.2).

Transitioning from a Parenteral DTI

After initial therapy with a non-heparin parenteral anticoagulant, HIT patients are usually transitioned to oral anticoagulation (OAC) for longer-term treatment in the outpatient setting. Direct oral anticoagulants (DOACs), such as dabigatran, apixaban, rivaroxaban, and edoxaban, have not been extensively studied in HIT, and thus warfarin remains the preferred OAC in this setting. The ACCP guidelines recommend starting warfarin once the platelet count has recovered to $>150 \times 10^3$/microliter (or to patient's baseline) and to continue overlap with the chosen parenteral non-heparin anticoagulant for ≥5 days and until INR is within a target range for a period of time during the overlap. The INR should then be rechecked after discontinuation of the parenteral anticoagulant to determine an INR based solely on the warfarin [13].

Argatroban

Argatroban significantly prolongs the PT/INR, which makes transitioning to warfarin a challenging process. Argatroban labeling does not recommend a specific duration of overlap; however, it recommends to continue the overlap with a goal INR of >4 if the argatroban infusion rate is ≤2 mcg/kg/min. For rates >2 mcg/kg/min, it is recommended to temporarily reduce the rate to 2 mcg/kg/min to determine the INR at that rate. However, if a patient has required infusion rates >2 mcg/k/min in order to maintain a goal INR, decreasing the rate may put the patient at risk of undertreatment. Thus, it is suggested that providers may use the chromogenic factor X activity assay if available (not to be confused with an anti-FXa LMWH or UFH assay) to monitor warfarin during the transition with a goal target range of 20–40% factor X activity (corresponds to an INR of 2–3). Unfortunately, this assay is often not readily available or is a send-out lab with a prolonged turnaround time that is not conducive to acute care. An alternative approach on days 4 or beyond (when warfarin should begin to have some effect) or once the INR is >4–5, one can shut off the argatroban for 2 h and then collect a blood sample for an accurate INR that reflects warfarin activity alone. The argatroban infusion should be resumed as soon as possible once the INR is drawn and continued while awaiting results to avoid any significant gaps in therapy.

Bivalirudin

Bivalirudin also impacts the INR but much less so than argatroban [32]. Aiming for an INR goal of 1.0 greater than the planned warfarin target INR is likely sufficient, without need

for interruption of bivalirudin therapy. For example, bivalirudin and warfarin should be overlapped ≥5 days and until the INR is >3 for at least 24 h.

Managing Bleeding and Reversal

Adverse Events

Bleeding is the most serious adverse effect associated with the parenteral DTIs. Concomitant use of other antithrombotics (antiplatelet agents, etc.) poses a pharmacodynamic interaction that may potentiate bleed risk. General approaches to bleeding management (e.g., looking for and controlling the source of the bleed) and supportive measures, such as resuscitation and monitoring, should be employed. There are no specific antidotes for argatroban or bivalirudin. Cessation of the infusion during recognition of a bleed is the most prudent intervention given their short half-lives and rapid elimination from the body [15]. In healthy subjects, coagulation parameters return to baseline 1–2 h after stopping a parenteral DTI infusion. The ACCP guidelines suggest that activated recombinant factor VIIa may potentially be used to reverse the effects of argatroban and bivalirudin in urgent situations, but this has not been studied in humans [1].

Oral Anticoagulants

Vitamin K Antagonists

Overview

Dicoumarol, the first vitamin K antagonist (VKA), was isolated from spoiled sweet clover in 1939–1940 by Professor Karl Paul Link [33]. Warfarin was subsequently synthesized (1942) and developed as a rodenticide but then went into clinical use in the 1950s. The VKAs were the only oral anticoagulants until the development of the direct oral anticoagulants (DOACs) introduced in 2010. Warfarin is the major VKA worldwide and the principal formulation used in North America. Other formulations with different pharmacokinetics are in use outside of the United States. Approximately 1–2% of the population of first-world countries is prescribed an oral anticoagulant. The VKAs work indirectly by interfering with the synthesis of the vitamin K-dependent coagulation factors (factors II, VII, IX, and X) leading to impaired coagulation (Fig. 17.4) [34]. The VKAs have many drawbacks, require considerable dose management to maintain a patient in a therapeutic range, and result in undertreatment as a consequence.

Key Concepts

- The vitamin K antagonists inhibit the synthesis of the vitamin K-dependent coagulation factors II, VII, IX, and X. Warfarin is the most common vitamin K antagonist.
- Warfarin therapeutic levels are affected by multiple factors including diet, drugs, genetics, and concomitant illnesses and, as such, require careful and frequent monitoring and dose adjustment.
- The prothrombin time, reported as an international normalized ratio (INR), equilibrates results from different thromboplastin reagents and is the common measure of therapeutic efficacy.
- Specialized programs referred to as anticoagulation clinics provide the most effective therapy by patient education, focused proactive dose management, and keeping patients within a specified therapeutic range.

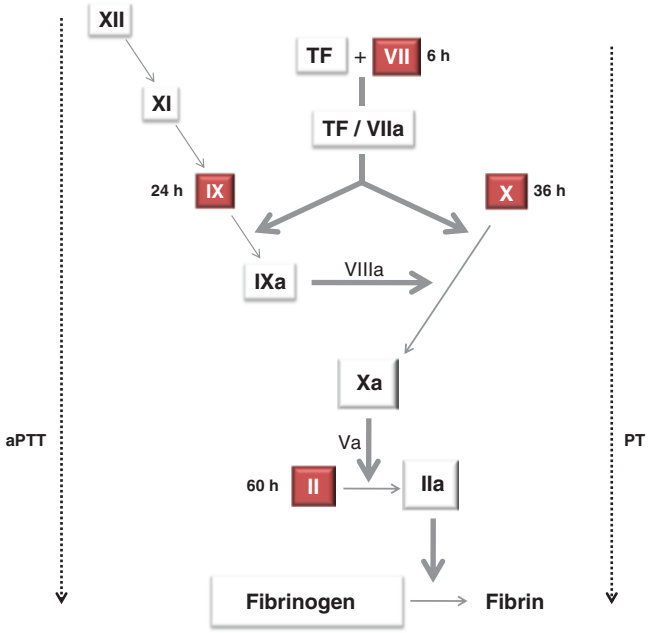

Fig. 17.4 Simplified scheme of the coagulation cascade indicating those factors that are vitamin K dependent for their normal synthesis (shaded) and their natural metabolic half-lives. Warfarin interferes with vitamin K-dependent factor synthesis; its effect on the coagulation cascade can be measured by the prothrombin time (PT) as well as the activated partial thromboplastin time (aPTT), although the PT is the more responsive and clinically useful measure of warfarin's effect

Pharmacology

Mechanism of Action

Vitamin K is an essential cofactor in the posttranslational γ-carboxylation of several glutamic acid residues in the vitamin K-dependent coagulation factors II, VII, IX, and X (Fig. 17.5), as well as proteins C and S [34, 35]. With reduced or absent γ-carboxylation, these proteins are unable to bind cell membranes, calcium, and phospholipid, and they manifest a reduced coagulant or enzymatic activity. Warfarin produces its anticoagulant effect by interfering with the cyclic interconversion and regeneration of reduced vitamin K from its 2,3-epoxide (oxidized vitamin K) by inhibiting the enzyme vitamin K oxide reductase complex 1 (VKORC1) responsible for this interconversion (Fig. 17.5). The enzyme,

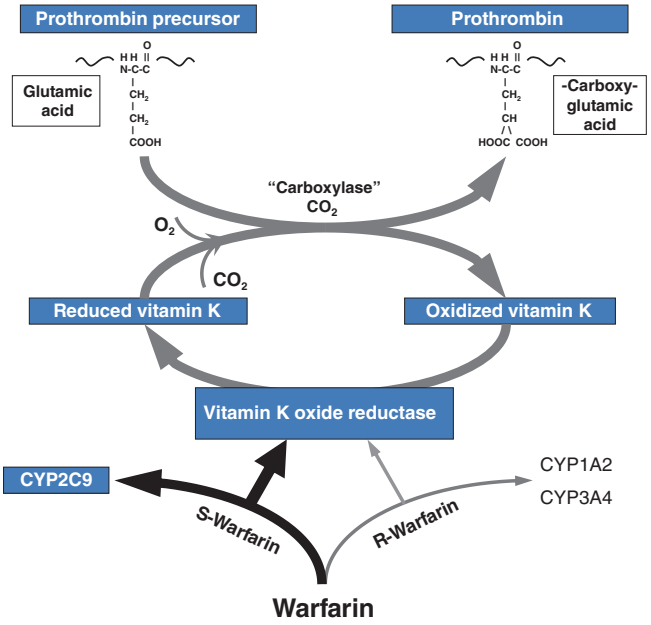

Fig. 17.5 The vitamin K cycle. Reduced vitamin K participates in a carboxylation reaction to gamma carboxylate a number of glutamic acid moieties in the prothrombin precursor leading to the synthesis of functional prothrombin. In the process, reduced VK is oxidized. The latter moiety is recycled via a vitamin K oxide reductase enzyme (VKOR). VKOR is the target of warfarin (mainly the S isomer of warfarin) leading to an accumulation of oxidized vitamin K. S-warfarin is metabolized mainly by the liver cytochrome P450 enzyme, 2C9 [78]

carboxylase, then utilizes reduced vitamin K along with oxygen and CO_2 to create carboxyglutamic acid on the vitamin K proteins to prepare the ideal protein forms to participate in the kinetically fast hemostatic reactions of tenase and prothrombinase (see Chap. 10).

Dietary vitamin K enters the body in a partially reduced state, bypassing the warfarin-sensitive reductase and replenishing fully reduced vitamin K stores in the presence of warfarin therapy. In response to warfarin, the vitamin K-dependent factors decline according to their natural half-lives with factor VII declining most rapidly (~ 6 h) and prothrombin (factor II) least rapidly (~ 60 h) (Fig. 17.4). Factor IX has a half-life of 24 h; factor X has a half-life of 48–60 h. The prothrombin time, a measure of the extrinsic and common pathways of coagulation, is most sensitive to this change and is initially influenced by the rapid fall of factor VII (see Chap. 11).

Pharmacokinetics/Pharmacodynamics

Warfarin is highly water soluble, rapidly absorbed from the gastrointestinal tract with peak absorption in 60–90 min and highly protein bound by albumin after absorption. Warfarin is a racemic mixture of stereoisomers (*R* and *S* enantiomers), each with distinctive metabolic pathways, half-lives, and potencies. Racemic warfarin has an average plasma half-life

$$\text{INR} = \left(\frac{\text{PT}}{\text{mean of normal}}\right)^{\text{ISI}}$$

Fig. 17.6 The derivation of the international normalized ratio (INR) from the prothrombin time ratio. The international sensitivity index (ISI) is derived by each manufacturer of thromboplastin by simultaneously comparing the PT result with their new thromboplastin with that from an international standard thromboplastin in 60 patients receiving warfarin therapy

of 36–42 h (range of 15–60 h). Differences in metabolism, disease- and/or drug-induced alterations in metabolic fate, or the sensitivity of the VKORC1 enzyme to warfarin account for much of the variation in an individual's initial response to, and maintenance requirement for, warfarin. The S enantiomer of warfarin (five times more potent than the R enantiomer) is metabolized primarily by the CYP 2C9 enzyme of the cytochrome P-450 (CYP450) system. A number of genetic polymorphisms (single-nucleotide polymorphisms, or SNPs) in this enzyme lead to a reduced activity of the enzyme and may influence both the dosage required to achieve a therapeutic level and the bleeding risk with warfarin therapy. Specifically, the *CYP2C9*2* and *CYP2C9*3* alleles are associated with lower-dosage requirements and higher bleeding complication rates compared with the wild-type *CYP2C9*1*. The prevalence of these polymorphisms varies in different populations. Renal impairment has no direct impact on warfarin since only its metabolites are excreted by the kidney and these have little or no anticoagulant activity. However, renal impairment may impair the function of CYP2C9 leading to accumulation of warfarin, thus enhancing its effect.

The "anticoagulant" effect of warfarin is related to a reduction of the vitamin K-dependent coagulation factors and is reflected by an elevation of the prothrombin time (PT) and the activated partial thromboplastin time (aPTT) (Fig. 17.4), both of which are most sensitive to reductions of factor VII and factor IX, respectively. However, the "antithrombotic" effect is most dependent on a reduction of prothrombin (factor II) and factor X in the common pathway of coagulation. The pharmacodynamic effect is most commonly measured by the PT as expressed as an international normalized ratio (INR), a derived result that corrects for different sensitivities of thromboplastin (tissue factor), the reagent used in the PT assay (see Fig. 17.6 for calculation of the INR) [36]. A safe and effective therapeutic INR range was first developed empirically but has since been confirmed by a number of large prospective trials [37, 38]. Table 17.3 summarizes the therapeutic range established for the different indications for which warfarin is used.

Table 17.3 The recommended indications for warfarin therapy and the recommended therapeutic range

Treatment of venous thrombosis	2.5 (2.0–3.0)
Treatment of pulmonary embolism	2.5 (2.0–3.0)
Prevention of systemic embolism	
Atrial fibrillation	2.5 (2.0–3.0)
Recurrent systemic embolism	2.5 (2.0–3.0)
After myocardial infarction[a]	3.0 (2.5–3.5)
Bioprosthetic heart valves (M or Ao position)[b]	2.5 (2.0–3.0)
Mechanical prosthetic heart valves	
Bileaflet valve in Ao position	2.5 (2.0–3.0)
Bileaflet or tilting valve in M position	3.0 (2.5–3.5)
Mechanical valve + atrial fibrillation (any position)	3.0 (2.5–3.5)
Mechanical valve + additional risk factors	3.0 (2.5–3.5) + aspirin (81 mg daily)

Abbreviations: *Ao* Aortic valve, *INR* international normalized ratio, *M* mitral valve

[a]For prevention of recurrent MI, an INR of 3.0 (2.5–3.5) is recommended

[b]For St. Jude or CarboMedics bileaflet or Medtronic-Hall tilting-disk valve

Warfarin pharmacodynamics is also dependent on the sensitivity of warfarin's target, the *VKORC1* enzyme. The *VKORC1 gene* also has a number of SNPs that lead to varying sensitivities of the enzyme to warfarin inhibition and have been shown to have a major impact on the pharmacodynamics of warfarin. A combination of SNPs leads to various haplotypes of the gene and gene product. Some of these haplotypes result in an enzyme that is more sensitive to warfarin inhibition so that a lower dosage of warfarin is required, whereas others are more resistant, so that a higher dosage (and maintenance dosage) of warfarin is needed to achieve a therapeutic INR. The prevalence of these haplotypes varies in different populations. A combination of genetic alterations in either the *CYP2C9* or *VKORC1* genes has been shown to account for as much as 20–50% of the variability in warfarin maintenance dosing, but as discussed below, pharmacogenetic testing is not routinely recommended to guide initiation or maintenance dosing.

Clinical Indications

The FDA-approved indications for warfarin include the following:

- Prophylaxis and treatment of venous thrombosis and its extension, pulmonary embolism
- Prophylaxis and treatment of thromboembolic complications associated with atrial fibrillation and/or cardiac valve replacement
- Reduction in the risk of death, recurrent myocardial infarction, and thromboembolic events such as stroke or systemic embolization after myocardial infarction

As primary prophylaxis for venous thromboembolism (VTE), warfarin is commonly used after major hip or knee replacement surgery, although other agents such as aspirin or DOACs are rapidly replacing warfarin for this indication. Similarly, for stroke prevention in atrial fibrillation, the DOACs are rapidly gaining on warfarin's market share for this indication. Warfarin remains the principal oral anticoagulant for patients with mechanical heart valves. Finally, warfarin is used to varying degrees after myocardial infarction, although the DOACs are also being studied for this indication. Warfarin has been the mainstay of treatment for the secondary prevention of VTE following an acute episode of VTE, but this indication is also being challenged by the DOACs.

Clinical Dosing and Management

Initiation of Therapy

During the initiation of therapy, warfarin has the potential to create a brief hypercoagulable state before it produces a hypocoagulable state. This is due to a reduction in protein C and S, both vitamin K-dependent proteins, the former of which has a short half-life similar to factor VII. As a result, protein C levels decline faster than prothrombin or factor X potentially leading to a hypercoagulable state. This is most problematic in patients who may already have low protein C levels. Consequently, in patients with an underlying thrombogenic condition such as a VTE, a rapid-acting anticoagulant such as heparin or low-molecular-weight heparin is started simultaneously with warfarin.

Warfarin therapy should be initiated using an average maintenance dose of about 5 mg for the first 2 or 3 days. Heparin or low-molecular-weight heparin (LMWH) should be given concurrently with warfarin when an immediate anticoagulant effect is required such as in the treatment of VTE, and treatment should overlap for a period of 4–5 days since it takes that long to lower levels of prothrombin and factor X, those factors mostly responsible for the antithrombotic effect of warfarin. Heparin is discontinued when the INR has been in the therapeutic range on two measurements taken at least 24 h apart. Using a 10 mg starting dose for outpatients is recommended by the 2012 American College of Chest Physicians (ACCP) Evidence-Based Clinical Practice Guidelines [35]; however, a recent Cochrane Database Systematic Review [39] of the optimal initial dose for warfarin failed to identify an optimal method or dosing schedule between an initial 5 and 10 mg dose. A starting dose lower than 5 mg may be appropriate in the elderly, in patients with impaired nutrition or liver disease, and in patients at high risk of bleeding.

Maintenance Dosing

Estimation of the maintenance dose is based on observations of the INR response following administration of a fixed dose

of warfarin over an interval of a few days [34]. An individual who rapidly achieves an elevated INR (above 1.5) after two doses of warfarin is likely to require a low maintenance dose. The opposite holds for a patient who shows little elevation of the INR (below 1.5) after two doses.

The patient's genotype for CYP2C9 and VKORC1 has been shown to influence dose (as much as 50% of the variability of the INR) and bleeding outcomes in a number of retrospective or observational trials, but prospective trials have shown mixed results for the benefit of pharmacokinetic [40, 41] dosing. In 1015 patients randomized to pharmacogenetic vs standard dosing, the North American COAG trial failed to show a difference in time-in-range between groups in the first 15 days or at 4 weeks or a significant difference in rates of the combined outcome of an INR of 4 or more, major bleeding, or thromboembolism [40]. Black patients managed by genotype-guided dosing actually had significantly less time-in-range than non-black patients managed by standard care. A number of other randomized controlled trials showed mixed results as have a number of systematic reviews [41].

The value of pharmacogenetic-guided dosing is limited by availability of rapid turnaround genetic assays, cost of genetic assays, complex algorithms needed to utilize genetic information, and conflicting trial results about the usefulness of such information and the overall cost-effectiveness. All of these must be compared with the relative in expense and value of frequent monitoring of a simple INR test. At the present time, pharmacogenetic-based dosing is not recommended by the ACCP guidelines [35].

Frequency of Monitoring

Monitoring is performed frequently when warfarin is initiated – every day or two until a therapeutic INR is achieved – and then the interval of monitoring is gradually extended depending on stability of dosing. Stable patients are usually monitored once every 4 weeks although some studies have shown that intervals of 6–12 weeks are safe in patients who are stable.

Managing Out-of-Range INRs

Out-of-range INRs are not uncommon. When an out-of-range INR is obtained, patients should be queried about diet (INR varies inversely with the amount of vitamin K in diet), medications (new starts or discontinuations), concomitant illnesses (e.g., heart failure, liver disease), and adherence to dosing. An elevated INR can be managed by briefly discontinuing warfarin, administering vitamin K, or infusing fresh-frozen plasma (FFP) or factor concentrates (Table 17.4). The choice is based largely on the severity of the clinical situation (e.g., degree of elevation of the INR, presence of severe bleeding). Assuming an ongoing normal food intake and reasonable hepatic function, when warfarin is stopped, it takes about 4–5 days for the INR to return to the normal range in patients whose INR is between 2.0 and 3.0. The INR will return to normal more quickly in patients requiring a larger daily maintenance dose than in those requiring a lower daily maintenance dose. Because the absolute daily risk of bleeding is low even when the INR is excessively prolonged, many physicians manage patients with INR values of 4.0–9.0 by simply holding warfarin and monitoring more frequently, unless the patient is at a higher risk of bleeding or bleeding has already developed. Vitamin K should be given orally or by the intravenous route. Intravenous injection rarely may be associated with anaphylactic reactions, but it does lead to

Table 17.4 Recommendations for managing elevated international normalized ratios (INRs) or bleeding in patients receiving warfarin

Condition*	Description
INR above therapeutic range but <5.0; no significant bleeding	Lower dose or omit dose, monitor more frequently, and resume treatment at lower dose when INR therapeutic; if only minimally above therapeutic range, no dose reduction may be required (Grade 2C)
INR ≥5.0 but <9.0; no significant bleeding	Omit next one or two doses, monitor more frequently, and resume treatment at lower dose when INR in therapeutic range. Alternatively, omit dose and give vitamin K (≤5 mg orally), particularly if at increased risk of bleeding. If more rapid reversal is required because the patient requires urgent surgery, vitamin K (2–4 mg orally) can be given with the expectation that a reduction of the INR will occur in 24 h. If the INR is still high, additional vitamin K (1–2 mg orally) can be given (Grade 2C)
INR ≥9.0: no significant bleeding	Hold warfarin therapy, and give higher dose of vitamin K (5–10 mg orally) with the expectation that the INR will be reduced substantially in 24–48 h. Monitor more frequently, and use additional vitamin K if necessary. Resume therapy at lower dose when INR is therapeutic (Grade 2C)
Serious bleeding at any elevation of INR	Hold warfarin therapy, and give vitamin K (10 mg by slow IV infusion), supplemented with fresh plasma or prothrombin complex concentrate, depending on the urgency of the situation; recombinant factor VIIa may be considered as alternative to prothrombin complex concentrate; vitamin K can be repeated every 12 h (Grade 1C)
Life-threatening bleeding	Hold warfarin therapy, and give prothrombin complex concentrate supplemented with vitamin K (10 mg by slow IV infusion); recombinant factor VIIa may be considered as an alternative to prothrombin complex concentrate; repeat if necessary, depending on INR (Grade 1C)

Abbreviations: *IV* Intravenous

*If continuation of warfarin therapy is indicated after high doses of vitamin K, then heparin or low-molecular-weight heparin can be given until the effects of vitamin K have been reversed and the patient becomes responsive to warfarin therapy. It should be noted that INR values of >4.5 are less reliable than values in or near the therapeutic range. Thus, these guidelines represent an approximate guide for high INRs

reversal of the INR more quickly than oral or subcutaneous administration of vitamin K. The response to subcutaneous vitamin K may be unpredictable and sometimes delayed. Ideally, vitamin K should be administered in a dose that will quickly lower the INR into a safe, but not subtherapeutic range, without causing resistance when warfarin is reinstated. High doses of vitamin K, although effective, may lower the INR more than is necessary and lead to warfarin resistance persisting for up to 1 week.

Outpatient management of warfarin therapy should aim for simplicity and clarity. It is recommended that a single or limited number of warfarin tablet strengths be used and that patients clearly understand the various dosing patterns that are used, such as alternate-day doses or dosing levels based on days of the week. One must also be aware that several different warfarin preparations are on the market, which can lead to confusion for the patient.

Managing Drug Interactions

Drug interactions commonly occur by affecting the pharmacokinetic or pharmacodynamic behavior of warfarin [42]. Drug interactions may interfere with GI absorption of warfarin or interfere with the metabolism of warfarin (via P450 2C9 hepatic enzyme) leading to a reduction or increase in clearance and, consequently, higher or lower plasma warfarin levels. The latter mainly affects the S enantiomer of racemic warfarin. Some drugs or disease states (liver disease, hyperthyroidism) can alter the metabolism of coagulation factors, inhibit coagulation factor interactions by other mechanisms (heparin), or inhibit other aspects of hemostasis (aspirin effect on platelet function) and lead to a greater risk of bleeding. Generally, interactions are most problematic when interacting drugs are added to or deleted from a patient's regimen, or a dose change is made. Once a patient has achieved stability on warfarin and an interacting medication, there should be little problem in maintaining stability of warfarin dosing. Managing drug interactions requires more frequent monitoring of the INR. This can be done prospectively when there is a planned drug addition or deletion and certainly when a patient presents with an abnormal INR resulting from a drug interaction or change in concomitant medications.

Dietary supplements and herbal preparations are also responsible for interacting with warfarin and may require special vigilance and questioning on the part of the clinician to reveal patient use of these products. For a listing of various drug interactions, the reader is referred elsewhere [42].

Managing Invasive Procedures

It is not uncommon for patients to require invasive procedures during warfarin therapy. Whether minor dental work or major surgery, the physician needs to assess the following questions [14]:

- Do I need to stop warfarin, i.e., what is the risk of bleeding during the procedure if warfarin is continued?
- If warfarin is stopped, do I need a short-acting anticoagulant to cover the pre- and post-procedure period, i.e., what is the risk of thromboembolism when warfarin is stopped for a few days?
- What is the risk of bleeding post-procedure with a short-acting anticoagulant?

Historically, clinicians often stopped warfarin and used bridging anticoagulation (LMWH) to protect against thromboembolism during the periprocedural period. Now, however, mounting evidence suggests that this is not needed and in fact may lead to an increase in bleeding in patients with low or moderate risk of thromboembolism off warfarin for a short period. There is not only a risk of major bleeding but also a risk associated with the delayed reinstitution of long-term therapy because of the bleeding. Investigators have shown a 3-month cumulative risk of major post-procedure bleeding of approximately 3% with bridging versus 1% without such bridging. In a recent study, 1884 patients with atrial fibrillation at low to moderate risk of thromboembolism off anticoagulants were randomized to either LMWH bridging or no [43] bridging. The outcome of arterial thromboembolism was the same in each group (0.3% vs 0.4%, respectively; $p = 0.01$ for non-inferiority). The incidence of major bleeding was 3.2% in the bridging group vs 1.3% in the no bridging group ($p = 0.005$). Similar results were found in a large retrospective analysis of 1178 Kaiser Permanente Colorado patients, mostly with venous thromboembolism as opposed to atrial fibrillation (AF) (2.7% major bleeding in bridged patients vs 0.2% in non-bridged patients). There was no difference in the rate of venous thromboembolism.

If bridging therapy is implemented in those at very high risk of thromboembolism, warfarin is usually discontinued 4 or 5 days before the procedure, and the INR is allowed to decline. LMWH is started 2 or 3 days before the procedure, usually at a full-treatment dose (100–150 anti-factor Xa U/kg subcutaneously) once or twice daily depending on the risk of thrombosis, with the last dose omitted the morning or night of the day before the procedure (approximately 12–24 h before the procedure). Most authorities recommend cessation of LMWH 24 h prior to surgery. It is then restarted about 12 h after the procedure along with warfarin. If the risk of postoperative bleeding from the procedure is high, LMWH can be restarted in 24–48 h, rather than in 12 h. When the INR becomes therapeutic, LMWH administration is stopped. Table 17.5 summarizes the author's approach integrated with various guideline recommendations for management of oral anticoagulation during invasive procedures.

Table 17.5 An approach for managing anticoagulation therapy in patients requiring an invasive procedure [78]

Risk of thromboembolism or bleeding	Example	Recommendation or suggestion[a]
Low risk of thromboembolism off anticoagulants with moderate to high risk of bleeding	Bileaflet mechanical aortic valve without AF; AF with $CHADS_2$ score < 2; VTE >12 month ago	Stop warfarin approximately 5 days before procedure; allow the INR to return to normal; no need to bridge with rapid-acting anticoagulant; resume warfarin after procedure
Intermediate risk of thromboembolism off anticoagulants with moderate to high risk of bleeding	Bileaflet mechanical aortic valve with other risk factors such as AF, prior stroke, heart failure, and hypertension; VTE 3–12 months ago; recurrent VTE; active cancer	Approach is based on individual patient and surgery-related factors. Based on results of the BRIDGE trial [76], bridging is generally not required. In some instances, one might use a prophylactic dose of LMWH
High risk of thromboembolism off anticoagulants with moderate to high risk of bleeding	Mechanical mitral valve; old-style aortic valve; AF with high $CHADS_2$ score (5 or 6); CVA/TIA <3 months; rheumatic valvular heart disease; recent (<3 month) VTE	Stop warfarin approximately 5 days before procedure; allow the INR to return to normal; begin therapy with full-dose UFH or LMWH approximately 3 days before procedure; stop UFH approximately 6 h before procedure; omit last one or two doses of LMWH; restart UFH or LMWH ~12–24 h after procedure or when patient is in hemostatically stable condition
Low risk of bleeding on anticoagulants	Dental work; screening colonoscopy; cataract surgery	Continue warfarin without adjusting dose or INR if within therapeutic range; alternatively omit a dose or two of warfarin and allow INR to fall to a lower range (~1.5–2.0) and restart after procedure

Note: These guidelines represent the author's approach and are an amalgam of guideline recommendations and recent clinical trial results. The risk of thromboembolism with discontinuation of anticoagulants vs the risk of bleeding during the procedure if anticoagulants are continued must be assessed for each patient; restarting a rapid-acting anticoagulant too soon after a procedure can lead to bleeding, which will then prolong resumption of warfarin anticoagulation and put the patient at risk of thromboembolism; deciding on a final course of action often requires a discussion with the physician performing the procedure

Abbreviations: AF Atrial fibrillation, *$CHADS_2$* system for scoring thromboembolic risk in non-valvular AF that assigns points for congestive heart failure, hypertension, age ≥75, diabetes mellitus, and prior stroke or transient ischemic attack, *CVA* cerebrovascular accident, *INR* international normalized ratio, *LMWH* low-molecular-weight heparin, *UFH* unfractionated heparin, *TIA* transient ischemic attack, *VTE* venous thromboembolism

[a]Based on recommendations of the American College of Chest Physicians Consensus Conference on Antithrombotic Therapy

Adverse Events: Managing Bleeding and Reversal of Therapy

Warfarin consistently ranks in the top three drugs to bring patients to an emergency room with an adverse event (hemorrhage), leading to hospitalization. In a recent report warfarin ranked #1 representing 15% of visits to an emergency room for a drug-related adverse event [44].

Patients with warfarin-related major bleeding must be supported hemodynamically (fluids and RBC transfusions if needed), receive intravenous vitamin K to maintain normal vitamin K-dependent factor levels by endogenous production, have their anticoagulation reversed (infusions of fresh-frozen plasma (FPP) or prothrombin complex concentrates (PCCs)), and lastly, have the site of bleeding identified and treated (if possible).

FFP has been the standard approach to correct a long INR for patients with bleeding on warfarin. The usual problem with FFP correction is that the tolerable dose is insufficient to correct the degree of defect. If FFP is used, it should be given at a dose of 15–30 mL/kg to have a significant impact on factor replenishment. However, many patients cannot tolerate that dose in the short period of time needed to correct a bleeding state or stop an intracerebral hemorrhage. PCCs are concentrates of the vitamin K-dependent factors (II, VII, IX, and X). Their principal advantage compared with FFP is their ability to restore normal coagulation in minimum time (15–30 min) without infusions of large volumes of FFP over hours which may also stress the heart in patients who may already be compromised [45]. Preparation time also is shorter than for FFP. PCCs do carry a small risk of enhancing the risk of thrombosis. Their efficacy has been studied prospectively in clinical trials [46]. PCCs are often dosed in a range of 25–50 IU/kg, but the specific dose will depend on degree of INR prolongation and desired level of correction.

Small studies have also shown that recombinant factor VIIa (rFVIIa) can reverse the coagulopathy and associated bleeding induced by warfarin, as well as other coagulopathies. However, how to dose has not been studied critically, and the range of dosing is reported empirically to range from approximately 20 μg/kg to more than 100 μg/kg.

Although factor concentrates are shown to return the INR to normal more rapidly than with FFP, there is still debate as to how beneficial they are over FFP in terms of outcome. Meta-analysis data suggest that they are efficacious [47, 48]. Both PCCs and rFVIIa have the potential to induce a prothrombotic state, and such thromboembolism has occurred as a result of therapy. Table 17.4 outlines the 2008 ACCP recommendations for managing patients receiving coumarin

Table 17.6 Comparative pharmacokinetics, pharmacodynamics, and other features of direct oral anticoagulants

	Dabigatran	Rivaroxaban	Apixaban	Edoxaban
Target(s)	IIa	Xa	Xa	Xa
Prodrug	Yes	No	No	No
Bioavailability (%)	6.5 (pH dependent)	80	50	62
Peak effect	1.5–3 h	2–4 h	1–3 h	1–2 h
Half-life[a]	12–17 h	5–9 h	9–14 h	10–14 h
Renal elimination (unchanged drug)	80%	33%	25%	50%
Protein binding (%)	35	90	87	55
Dialyzable	Yes	No	No	Possible
Interactions	P-gp	CYP 3A4, P-gp	CYP 3A4, P-gp	CYP 3A4 (minimal), P-gp
Monitoring	No	No	No	No
Dosing	Twice daily	Once daily	Twice daily	Once daily
Antidote	Idarucizumab	Andexanet alfa	Andexanet alfa	Andexanet alfa
		Ciraparantag (in development)	Ciraparantag (in development)	Ciraparantag (in development)
Lab measure	aPTT, TT, ECT	PT, Anti-Xa	Anti-Xa	Anti-Xa

Abbreviations: *P-gp* P glycoprotein, *3A4* cytochrome P450 3A4, *INR* international normalized ratio, *PT* prothrombin time, *aPTT* activated partial thromboplastin time, *TT* thrombin time, *ECT* ecarin clotting time
[a]Normal renal function

anticoagulants who need the INR lowered because of actual or potential bleeding. These recommendations have not significantly changed in the most recent guidelines.

Other adverse events rarely seen with warfarin therapy include a condition of acute skin and fat tissue necrosis due to the induction of a hypercoagulable state discussed above. Rarely, therapy can be associated with cholesterol crystal embolization to peripheral extremities resulting in a condition called purple toe syndrome.

Cessation of Therapy or Transition to Other Oral Anticoagulants

When a course of therapy has ended, warfarin can simply be stopped without a need for dose tapering. If the patient is to be transitioned to a DOAC, the general rule is to hold warfarin until the INR declines to approximately 2.0 and then start the DOAC. Because of its rapid onset of action, the latter will provide therapeutic anticoagulation within 2 h.

Systems of Anticoagulation Management

Because of the labor intensiveness of VKA management, specialized anticoagulation management services (clinics) have been established to handle large numbers of patients and to optimize dosing. These services are differentiated from usual care by their proactive and focused attention to warfarin dosing and patient education [49]. There is abundant evidence that these programs achieve a higher time in INR range and better outcomes [50]. With the development of point-of-care, handheld, INR monitors, patients are now able to monitor their own INR at home with dose management handled by their provider or, with proper training, manage their own dosing [51, 52].

Direct Oral Anticoagulants

Overview

Warfarin therapy presents the clinician with several management problems in that the therapeutic level is affected by many factors, including diet, other medications, illnesses, and genetics. Therapy is hampered by a high degree of poor treatment with only approximately 50% of patients maintained in a therapeutic INR during therapy resulting in a high incidence of bleeding or thrombosis. The new direct oral anticoagulants are free of many of warfarin's drawbacks. These drugs include a direct thrombin (IIa) inhibitor (dabigatran etexilate) and three direct Xa inhibitors (apixaban, edoxaban, and rivaroxaban) (Table 17.6). When these factors are inhibited, fibrin and clot formation are impaired (Fig. 17.7). These small molecules directly block either factor Xa or thrombin (factor IIa) at their catalytic pocket. They promise to be more convenient and possibly safer than warfarin because they are given in fixed dosages, have a predictable anticoagulant effect, do not require monitoring, have few or minimal interactions with drugs or diet, and have a rapid onset of action that eliminates the need for parenteral anticoagulation in many situations.

Key Concepts

- The direct oral anticoagulants (DOACs) are small molecules that bind to the active enzymatic pocket of their specific target (either factor IIa or Xa) and neutralize the target, thus impairing coagulation.
- The DOACs have favorable pharmacokinetics that make them easier to manage than the vitamin K antagonists including a rapid onset and offset of action, predictable dose response, and few or no interactions with drugs or diet.

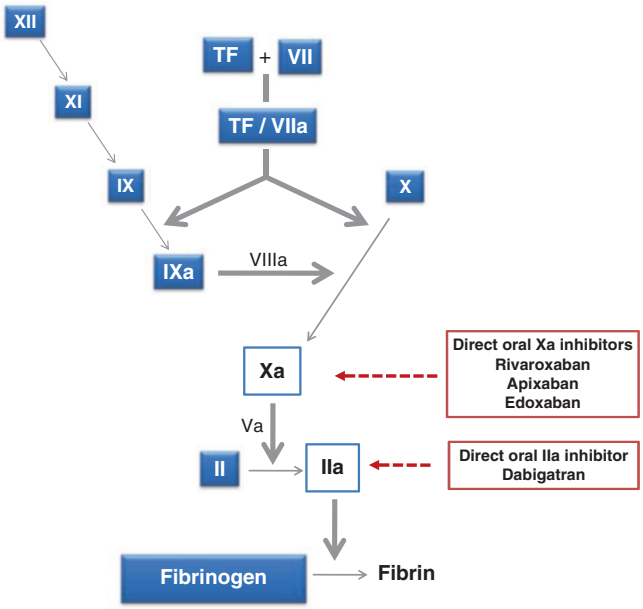

Fig. 17.7 Site of action of the direct oral anticoagulants. *TF* tissue factor

- The DOACs are all dependent to a greater or lesser extent on renal elimination of unchanged drug so that knowledge of renal function is important when initiating DOAC therapy.
- DOACs have been compared to the VKAs in large clinical trials of stroke prevention in atrial fibrillation and for the treatment and prevention of venous thromboembolism. In all cases, they have been found to be as effective as the VKAs and, overall, safer than the VKAs, especially with regard to intracranial bleeding.

Pharmacology

Mechanism of Action/Pharmacokinetics/Pharmacodynamics

Dabigatran etexilate (Pradaxa®, Boehringer Ingelheim) is a direct, selective, and oral thrombin inhibitor administered once or twice daily [53]. Dabigatran etexilate is an oral prodrug that is rapidly converted by a serum esterase to dabigatran, a potent, direct, competitive thrombin inhibitor. By inhibiting thrombin, it interferes with the fibrinogen to fibrin reaction and clot formation. It also impairs the activation of platelets by thrombin. Dabigatran has a 2-h onset of action, 14–17-h half-life, and 6.5% bioavailability. Eighty percent of dabigatran is excreted via the kidney unchanged, and renal function is an important determinant of therapeutic levels. Table 17.6 reviews dabigatran's relevant properties.

Rivaroxaban is a direct, selective, and oral factor Xa inhibitor administered mostly once daily [53]. It has an onset of action of 2–4 h and a half-life of 5–9 h and is partially eliminated by the kidney (approximately 33% unchanged drug) with the remainder metabolized by CYP450 enzyme 3A4. By binding to activated factor Xa, rivaroxaban interferes with Xa's ability to catalyze the conversion of prothrombin to thrombin and leads to impaired clot formation. Rivaroxaban has no effect on platelet function. Table 17.6 summarizes rivaroxaban's relevant properties.

Apixaban is a direct, selective, and oral factor Xa inhibitor administered twice daily [53]. It has a 1–3 h onset and 9–14 h half-life. Approximately 25% of apixaban is eliminated via the kidney with the rest metabolized by CYP 3A4. Similar to rivaroxaban, apixaban interferes with factor Xa's ability to generate thrombin from prothrombin. It has no effect on platelet function. Table 17.6 reviews apixaban's relevant properties.

Edoxaban is a third direct, selective, and oral factor Xa inhibitor administered once daily [53]. Similar to the other Xa inhibitors, it has a rapid onset of action within 1–2 h after oral administration with a half-life of approximately 10–14 h. Edoxaban has negligible CYP 450 metabolism and approximately 50% is excreted unchanged by the kidney. It impairs coagulation similar to the other factor Xa inhibitors. Table 17.6 reviews edoxaban's relevant properties.

Clinical Indications

The FDA-approved indications for the four DOACs are as follows:

Dabigatran Etexilate

- To reduce the risk of stroke and systemic embolism in patients with non-valvular atrial fibrillation
- For the treatment of deep venous thrombosis (DVT) and pulmonary embolism (PE) in patients who have been treated with a parenteral anticoagulant for 5–10 days
- To reduce the risk of recurrence of DVT and PE in patients who have been previously treated
- For the prophylaxis of DVT and PE in patients who have undergone hip replacement surgery

Rivaroxaban

- To reduce the risk of stroke and systemic embolism in patients with non-valvular atrial fibrillation
- For the treatment of DVT and PE and for the reduction in the risk of recurrence of DVT and of PE
- For the prophylaxis of DVT, which may lead to PE, in patients undergoing knee or hip replacement surgery

Apixaban

- To reduce the risk of stroke and systemic embolism in patients with non-valvular atrial fibrillation
- For the treatment of DVT and PE and for the reduction in the risk of recurrent DVT and PE following initial therapy

- For the prophylaxis of DVT, which may lead to PE, in patients who have undergone hip or knee replacement surgery

Edoxaban

- To reduce the risk of stroke and systemic embolism (SE) in patients with non-valvular atrial fibrillation. Edoxaban should not be used in patients with creatinine clearance (CrCL) >95 mL/min because of increased risk of ischemic stroke compared to warfarin at the highest dose studied (60 mg).
- For the treatment of DVT and PE following 5–10 days of initial therapy with a parenteral anticoagulant.

Clinical Dosing and Management

Dosing for VTE, AF, Orthopedic Surgery

Based on the results of large randomized clinical trials [54–62], DOACs are now recommended as first-line therapy for stroke prevention in AF and for the treatment of acute VTE. However, individuals with certain characteristics should not be considered for DOAC therapy. Table 17.7 summarizes general and specific criteria that should be considered in prescribing a DOAC.

Table 17.7 Considerations for selection of patients for direct oral anticoagulant therapy with atrial fibrillation or venous thromboembolism

Indication	Patient selection	Clinical considerations that may influence choice of therapy or exclude therapy with a DOAC
Stroke prevention in non-valvular atrial fibrillation	All patients with non-valvular AF, persistent, intermittent, or paroxysmal meeting specific risk factor criteria Non-valvular AF excludes patients with mitral stenosis or artificial heart valves and/or valve repair	Significant drug-drug interactions
		Significant renal impairment
		Significant liver impairment
		History of gastrointestinal bleeding
		Mechanical heart valve (excludes use of DOACs)
		Pregnancy/breast feeding (excludes use of DOACs)
		Extremes of body weight
		History of poor drug adherence
		Inability to pay for drug
Treatment of acute venous thromboembolism	All patients with VTE	Above considerations plus:
Prevention of recurrent VTE for extended treatment		Strong thrombophilic state
		Antiphospholipid syndrome
		Active cancer

Each DOAC has specific dose and dose modification recommendations for their major indications. These are summarized in Table 17.8. For the treatment of acute VTE, both rivaroxaban and apixaban can be started at the time of diagnosis without a lead-in phase with a parenteral anticoagulant (i.e., heparin or LMWH). However, both drugs are started at a higher dose for an initial period (Table 17.8) and then de-escalated to a lower maintenance dose for the duration of therapy. Both edoxaban and dabigatran require a lead-in phase of heparin or LMWH for at least 5 days before the DOAC is started based on the design of their clinical trials.

Monitoring Therapy and Coagulation Assays

Routine monitoring of the anticoagulant effect of DOACs is not necessary because they have a wide therapeutic window and their dose effect is predictable and is minimally influenced by drugs or diet. However, one would want to know their impact on coagulation in situations of major bleeding, trauma, or emergent surgery. In such situations a widely available, sensitive, and rapid turnaround assay would be desired [63]. Unfortunately, such assays are not clinically available. Table 17.9 summarizes both qualitative and quantitative assays that reflect drug concentration and/or drug effect on the coagulation cascade [64]. In clinical situations, the manufacturers currently recommend aPTT for measuring the effect of dabigatran and PT to determine the anti-Xa activity of rivaroxaban, apixaban, and edoxaban. Neither of these assays is ideal, all are reagent-dependent in their ability to reflect drug activity, and none are sensitive enough to exclude the presence of drug. For dabigatran, only the thrombin time (TT) is sensitive enough to exclude significant drug present when it is normal. The TT is not as widely available as the other assays noted above, and it may not be available during the 24 h cycle in those hospitals that do provide a TT. Thus, in patients taking dabigatran, an elevated aPTT suggests meaningful levels of dabigatran, but a normal aPTT cannot definitively exclude important levels of drug onboard.

The PT is more sensitive than the aPTT for assessing the effect of the factor Xa inhibitors and is recommended by the manufacturers. Like the aPTT, the result is reagent dependent and is most sensitive to rivaroxaban and much less sensitive to apixaban. The chromogenic anti-factor Xa assay with appropriate calibrators for each drug is an excellent quantitative assay, but it has very limited on-site availability in US hospitals, and the turnaround time from reference laboratories is obviously not suitable for emergent situations. Thus, the PT is helpful if elevated without other causes for elevation, but a normal PT does not rule out drug on board.

Table 17.8 Dosing of direct oral anticoagulants (DOACs) for stroke prevention in atrial fibrillation and treatment of acute and extended VTE

	Atrial fibrillation		Treatment of acute VTE		Extended VTE treatment	Joint replacement
Drug	Standard dosing	Modified dosing	Standard dosing	Modified dosing	Standard dosing	Standard dosing
Dabigatran	150 mg twice daily 110 mg twice daily (CrCl >30 ml/min)[a]	75 mg twice daily (CrCl 15–30 ml/min) (in the United States only)	150 mg twice daily (after 5–10 day heparin lead-in) (CrCl >30 ml/min)	None	150 mg twice daily CrCl <50 ml/min avoid use of concomitant P-gp inhibitor	110 mg for first day, then 220 mg once daily (CrCl >30 ml/min)
Rivaroxaban	20 mg once daily (CrCl >50 ml/min) with evening meal	15 mg once daily (CrCl 15–50 ml/min) with evening meal	15 mg twice daily for first 21 days and then 20 mg once daily with food	None (no renal dose adjustment)	20 mg once daily with food	Hip: 10 mg once daily for 35 days Knee: 10 mg once daily for 12 days
Apixaban	5 mg twice daily	2.5 mg twice daily with any two of following: age ≥80y; weight <60 kg; ser Cr ≥1.5 mg/dL	10 mg twice daily for 7 days and then 5 mg twice daily	None (no renal dose adjustment)	2.5 mg once daily after >6 months treatment of acute VTE	Hip: 2.5 mg twice daily, 12–24 h after surgery for 35 days Knee: 2.5 mg twice daily, 12–24 h after surgery for 12 days
Edoxaban	60 mg once daily	Half dose if CrCl 30–50 ml/min; wt <60 kg or with potent P-gp inhib; not used CrCl >95	60 mg once daily (after 5-day heparin lead-in)	30 mg once daily with a CrCl 30–50 ml/min or weight <60 kg or with potent P-gp inhib	None	None

[a]110 mg dosing not FDA approved in the United States

Table 17.9 Assays to measure effect of DOACs

Clinical objective					
	Determine if clinically relevant below on-therapy drug levels are present		Estimate drug levels within on-therapy range	Determine if above on-therapy drug levels are present	
Drug	Suggested test	Interpretation	Suggested test	Suggested test	Interpretation
Dabigatran	TT	Normal TT likely excludes clinically relevant drug levels	Dilute TT, ECA, ECT	aPTT, dilute TT, ECA, ECT	Normal aPTT likely excludes excess drug levels; only dilute TT, ECA, and ECT are suitable for quantitation
Rivaroxaban	Anti-Xa	Normal anti-Xa activity likely excludes clinically relevant drug levels	Anti-Xa	Anti-Xa, PT	Normal PT likely excludes excess drug levels; only anti-Xa is suitable for quantitation
Apixaban	Anti-Xa	Normal anti-Xa activity likely excludes clinically relevant drug levels	Anti-Xa	Anti-Xa	
Edoxaban	Anti-Xa	Normal anti-Xa activity likely excludes clinically relevant drug levels	Anti-Xa	Anti-Xa, PT	Normal PT likely excludes excess drug levels; only anti-Xa is suitable for quantitation

Abbreviations: *aPTT* activated partial thromboplastin time, *ECA* ecarin chromogenic assay, *ECT* ecarin clotting time, *PT* prothrombin time, *TT* thrombin time

Managing Invasive Procedures

With the rapid onset and offset of action of the DOACs, the management of patients requiring invasive procedures during therapy is greatly simplified compared to the VKAs. Analyses of interruptions of therapy in the major AF clinical trials of DOACs show that outcomes with the DOACs are no different than outcomes with warfarin, whether bridging is used or not. When a procedure is associated with little or no bleeding, there is no reason to interrupt DOAC therapy. If mild to moderate bleeding from the procedure is a potential, then skipping doses equivalent to 2–3 half-lives is recommended. For procedures that might incur major bleeding, one should skip 4–5 or more half-lives of the DOAC. In this calculation, it is important to be aware of renal function,

which, if impaired, may significantly prolong the half-lives of the DOACs, especially dabigatran. Restarting the drug post-procedure should be done when there is hemostatic stability, usually within 12–24 h depending on the procedure. It is important to remember that re-anticoagulation occurs almost immediately with the DOACs compared to the several days it takes with reinitiation of warfarin therapy.

Managing Drug Interactions

Drug-drug interactions with the DOACs occur either by induction or inhibition of the P-glycoprotein (P-gp) drug transporter (for dabigatran, rivaroxaban, apixaban, and edoxaban) or by induction or inhibition of the CYP450 3A4 metabolic enzyme (only Xa inhibitors with rivaroxaban being most sensitive, apixaban less sensitive, and edoxaban essentially not sensitive). There are many fewer important drug interactions with DOACs compared to those with VKAs, but when a potential or known interaction is present, the management difficulty lies in not knowing how much the interaction impacts DOAC drug concentration and inhibition of coagulation because there is no simple assay to accurately measure this effect. Generally, strong P-gp inhibitors and inducers should be avoided with dabigatran, especially in the setting of renal impairment. For the Xa inhibitors, it is recommended to avoid combined P-gp and strong CYP 3A4 inducers or inhibitors. The package inserts for each drug contain prescribing information for drug interactions. For some of the DOACs, dose reduction is suggested (e.g., dabigatran and apixaban) in the setting of certain drug-drug interactions, and for others (rivaroxaban and edoxaban) avoidance is suggested.

Adverse Events: Managing Bleeding and Reversal of Therapy

Besides maintaining hemodynamic balance with fluids and red cell transfusions and identifying the site of bleeding and treating it directly, if possible, managing bleeding in patients on DOACs also requires knowledge of which DOAC the patient is taking and when the last dose was taken, assessing renal function and trying to assess the degree to which the coagulation system is impaired [63, 65]. Lastly, for major bleeding, especially life-threatening bleeding, reversal of DOAC activity should be implemented. Although major bleeding with the DOACs still occurs at a rate of 1–3%, the DOACs are associated with less major bleeding, especially intracranial bleeding, compared to traditional therapy. Most minor or even moderate bleeding episodes are easily managed with a wait-and-see strategy allowing the drug effect to wear off. Knowledge of renal function impairment is particularly important for patients on dabigatran and to a somewhat lesser extent for edoxaban, the two DOACs with the highest rate of renal excretion. Assessing residual drug effect by the PT or aPTT assays (or TT for dabigatran) may be helpful, but, as previously discussed, normal assays do not fully exclude the presence of drug. For major life-threatening bleeding such as intracranial bleeding, bleeding into a vital organ, or hemodynamically unstable GI bleeding, reversal of the anticoagulant effect (if any remaining) should be attempted.

Idarucizumab is a specific reversal agent for dabigatran [66]. Andexanet alfa is a specific reversal agent for the factor Xa inhibitors [67]. A third reversal agent, ciraparantag, is currently under development [68]. The off-label use of PCCs as a prothrombotic agent to counter the anticoagulant activity of the factor Xa inhibitor is an alternative to andexanet alfa. This use is recommended only in life-threatening bleeding.

Idarucizumab (Praxbind®, Boehringer Ingelheim) is a murine monoclonal antibody that reverses the anticoagulant activity of dabigatran in patients with major bleeding or needing urgent surgery, as demonstrated in a recent clinical trial. Idarucizumab was able to normalize clotting times (dilute thrombin time or ecarin clotting time) in almost all patients within minutes of infusion which remained normal for the subsequent 24 h [66]. Ninety-three percent of patients who required emergent surgery experienced normal hemostasis during surgery as reported by their surgeon. The dose of idarucizumab is 5 gm given as sequential 2.5 gm intravenous injections.

Andexanet alfa (Portola Pharmaceuticals) is a unique recombinant human factor Xa that is modified so as to have no procoagulant effect but still retain binding specificity for the Xa inhibitors. It was recently approved by the FDA for clinical use to reverse anticoagulation in patients with major or life-threatening bleeding related to anticoagulation with rivaroxaban or apixaban. It needs to be given as a continuous infusion (given over 2 h in clinical trials) to maintain normal coagulation since its effect dissipates rapidly and anticoagulation will return based on the residual level of the DOAC. In two clinical trials, andexanet alfa was shown to rapidly reverse the anticoagulant effect of apixaban and rivaroxaban in healthy elderly volunteers and to restore adequate hemostasis within 12 h of administration in approximately 80% of patients who present with major bleeding [67].

Lastly, ciraparantag (Perosphere Inc.) is a small synthetic compound that binds to and neutralizes apixaban, rivaroxaban, and edoxaban as well as dabigatran and the indirect Xa inhibitor, enoxaparin [68]. Early studies indicate that a single intravenous injection can reverse anticoagulation induced by the abovementioned DOACs and enoxaparin with reversal persisting for up to 24 h. Ciraparantag is in the early stages of development and phase three trials have not yet been initiated.

Finally, other actions that might be suitable for select situations include activated charcoal lavage for patients who have recently ingested a DOAC (within 2–3 h) and, in the

case of dabigatran, hemodialysis. Dialysis is not suitable for the highly protein-bound Xa inhibitors.

Managing Transitions of Care

Care transitions, such as transferring from one medical institution to another or to home, are critical points in time where lack of a well-defined, organized, and efficient transition plan can result in adverse outcomes, loss of therapeutic effectiveness, and patient harm. Because DOACs have been promoted as drugs that do not need monitoring compared to the VKAs, there is the concern that patients will be left on their own with no oversight. For long-term outpatient follow-up, essential considerations include education about the drug and its potential side effects, the importance of drug adherence, dose reductions when applicable for the treatment of VTE, and periodic assessment of renal function, especially in those whose renal function is already impaired or who have concomitant illnesses that may accelerate decline.

Advantages/Disadvantages Compared with Warfarin

The DOACs have proven themselves to be as effective as traditional therapy for the major indications of stroke prevention in AF and the treatment of acute and chronic VTE. Perhaps more importantly, they have shown themselves to be safer than traditional therapy, especially for the incidence of intracranial hemorrhage. Because warfarin therapy has numerous drawbacks related to fluctuations in therapeutic range that require experienced and often labor intensive dose management, the DOACs are also attractive alternatives. With a wide therapeutic window, predictable dose response, and minimal influence by diet or other medications, they do not require routine monitoring. Their rapid onset of action eliminates the need for initial heparin therapy in many circumstances, and managing intervening invasive procedures is less complex. Table 17.10 summarizes the advantages of the DOACs. At the present time, the DOACs are rapidly gaining market share over the VKAs, although the latter will not totally go away. Meta-analysis data indicate that DOACs are as effective as warfarin in atrial fibrillation, deep venous thrombosis, and pulmonary embolism, but in all categories they have less bleeding [69]. However, there are certain indications for which the VKAs are still the mainstay of therapy, and in some circumstances, having a monitored, easily adjustable drug will have advantages for select patients.

Table 17.10 Characteristics of the direct oral anticoagulants (DOAC) versus warfarin

Characteristic	Warfarin	DOAC
Onset of anticoagulant effect	Slow	Rapid
Half-life	Long	Short (with normal renal function)
Dosing	Variable	Fixed (some variability)
Food effect	Yes	No
Drug interactions	Many	Few
Monitoring of anticoagulant effect	Yes	No
Antidote	Yes	Yes

Treatment of VTE

VTE treatment is divided into three phases: acute (first 5–10 days), long-term (first 3 months), and extended (>3 months). In the acute phase, the risk for adverse events such as deep vein thrombosis (DVT) extension, VTE recurrence, bleeding, and death is extremely high. Rapid attainment of therapeutic levels of anticoagulation is imperative to minimize short- and long-term morbidity and mortality.

Because of warfarin's slow onset, dosing strategies involving use of rapid-acting parenteral anticoagulants overlapped with warfarin evolved over time and have been shown to be extremely effective. With the advent of the direct oral anticoagulants (DOAC), approaches to VTE management have appreciably changed (Fig. 17.8). Based on their equal efficacy and improved safety compared to conventional approaches, DOACs are now preferred for treatment of VTE [24, 70]. It is important to emphasize that not all patients are DOAC candidates due to presence of contraindications or lack of evidence in certain populations. Thus, thoughtful patient selection is imperative for optimizing outcomes (Table 17.7).

When deciding on initial anticoagulation, the severity of presentation, potential need for invasive procedures, eligibility for outpatient treatment, and the patient's clinical characteristics, as well as their preferences, must all be considered (Fig. 17.9). Due to better safety and efficacy, the ACCP guidelines (9th ed. 2012) suggest either LMWH or fondaparinux (overlapped with warfarin) over UFH for initial management of VTE when a conventional approach is chosen. In general, unfractionated heparin should be reserved for use in specific clinical situations, such as patients with potential need for invasive procedures, potential for thrombolysis, or with increased bleeding risk, given its short half-life and complete reversibility with protamine sulfate. For VTE patients with severe renal impairment, UFH is preferred, as it is less reliant on renal elimination as compared to LMWHs, fondaparinux, and the DOACs. For all VTE patients who meet the criteria listed in Table 17.7, DOACs should be considered first-line therapy for treatment of VTE.

Special Populations/Considerations

Pregnancy and Breastfeeding

Warfarin is a known teratogen and the DOACs have not been studied in pregnant patients. Therefore, these agents should

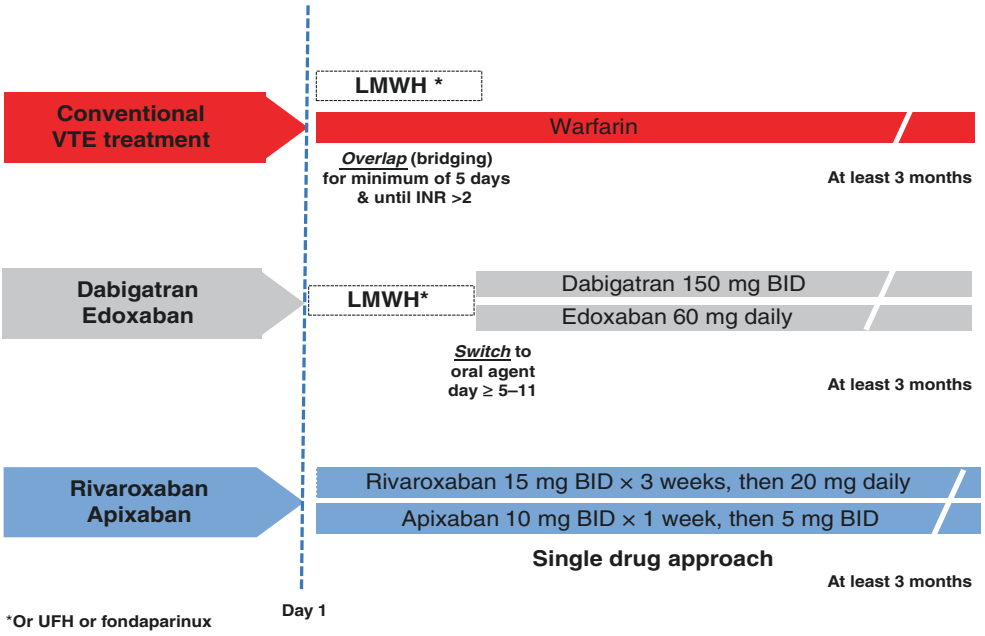

Fig. 17.8 Initiation of anticoagulation for acute VTE. BID twice daily, *INR* international normalized ratio, *LMWH* low-molecular-weight heparin, *UFH* unfractionated heparin, *VTE* venous thromboembolism

be avoided in pregnant patients with VTE. The exception to this is pregnant women with mechanical cardiac valves, in whom warfarin therapy may be considered during pregnancy. Fondaparinux is pregnancy category B and has been used successfully in pregnant patients with a contraindication to heparins. It may enter breast milk. However, due to its extensively proven safety and efficacy, the drug of choice for VTE in pregnancy is LMWH. If a pregnant patient has significant renal impairment, long-term SQ UFH therapy may be employed. There are no formal studies in humans evaluating the use of bivalirudin or argatroban during pregnancy, and use should be limited to situations that preclude the use of more conventional anticoagulants. They are relatively small molecules, and it is expected that they should cross the placenta. For breastfeeding, warfarin and LMWH are preferred therapies. The safety of parenteral DTIs, fondaparinux, or DOACs has not been established in breastfeeding women, and they should be avoided.

Cancer

Among patients with active malignancy and acute VTE, LMWH monotherapy for the first 3–6 months is preferred based on data from clinical trials showing superior efficacy and safety. Meta-analyses suggest DOACs are as efficacious as warfarin in preventing VTE recurrence; however, the number of patients with active cancer that were treated with DOACs in the clinical trials is small, and it is unknown if they are comparable to LMWH for this indication. Two prospective randomized clinical trials of ~1500 patients indicate that DOACs are equivalent to LMWH (dalteparin) in managing thrombosis in cancer patients [71, 72]. If a patient adamantly refuses long-term LMWH injections or warfarin plus frequent monitoring, use of DOACs may be considered (Fig. 17.9).

Thrombophilias

The DOACS have not been specifically studied in inherited or acquired thrombophilia. It is likely that a number of patients with an undiagnosed thrombophilia were enrolled in the DOAC VTE trials, suggesting these agents may be a viable option in this population. There are anecdotal reports that patients with a strong thrombophilic condition such as antiphospholipid syndrome do not do well with the DOACS. Until more robust data is available, a conventional approach with LMWH plus warfarin titrated to an INR of 2–3 is recommended (Fig. 17.9).

Extremes of Weight

The DOAC VTE trial populations did not adequately represent patients at extremes of weight. For patients <40 kg or >120 kg, it is unknown if fixed-dose DOACs might lead to over- or undertreatment in these patients. A conventional approach with LMWH (without dose capping in obesity) or fondaparinux (without need for dose adjustment in obesity) overlapped with warfarin is currently recommended until more data and experience are available (Fig. 17.9).

Renal Impairment

The LMWHs, fondaparinux, and the DOACs are all renally eliminated to an appreciable degree and thus should be avoided in severe renal impairment (estimated CrCl <30 ml/min). Preferred therapies in this population include UFH for acute management, with transition to warfarin for longer-term therapy. Either argatroban or dose-adjusted

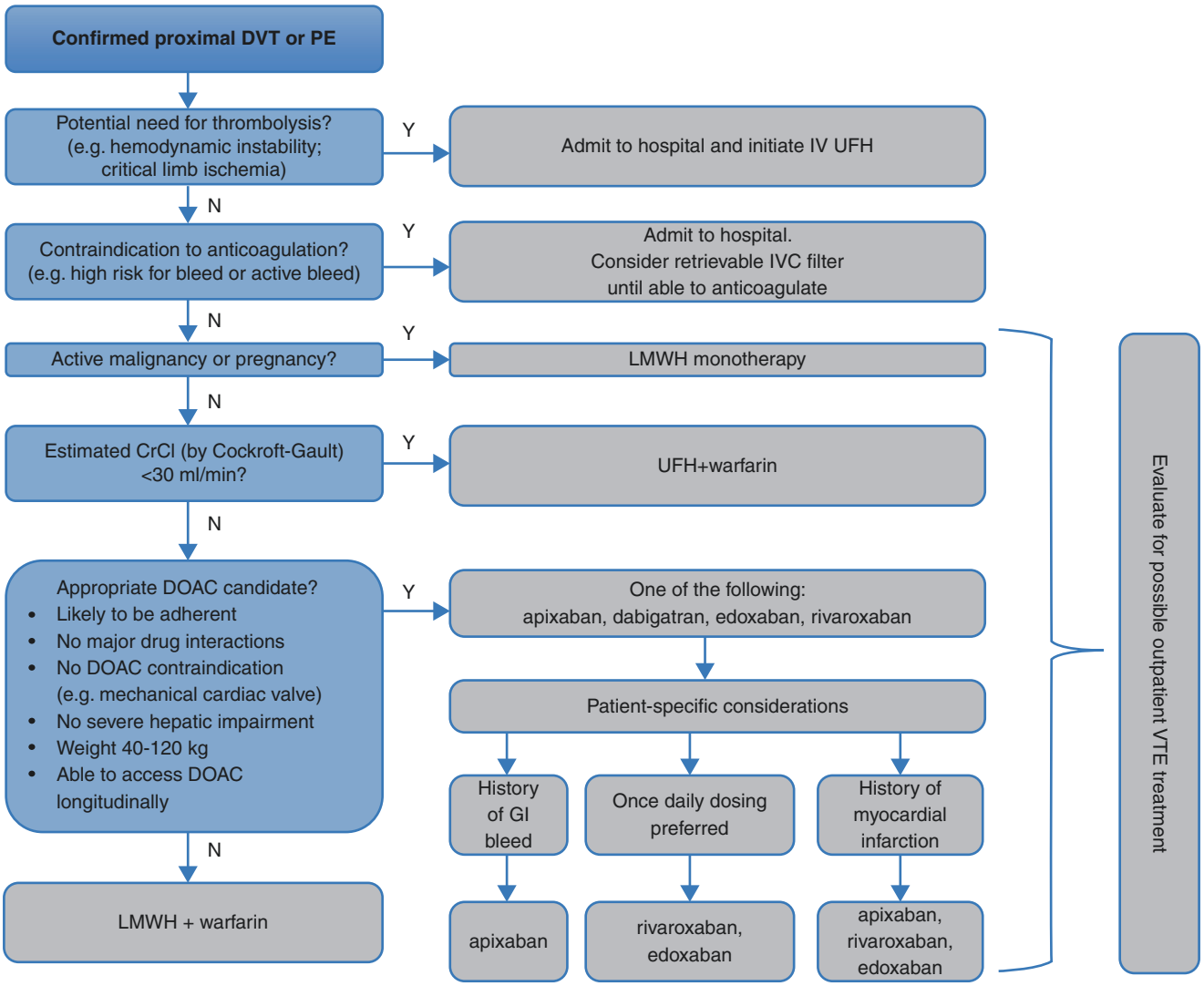

Fig. 17.9 Selection of anticoagulant for VTE treatment. *DOAC* direct oral anticoagulant, *DVT* deep vein thrombosis, *GI* gastrointestinal, *IVC* inferior vena cava, *LMWH* low-molecular-weight heparin, PE pulmonary embolism, *UFH* unfractionated heparin, *VTE* venous thromboembolism

bivalirudin may be used in patients with severe renal impairment requiring a parenteral DTI (Fig. 17.5).

Pediatrics

Warfarin, UFH, and LMWH have been used extensively in the pediatric population for a variety of indications. The evidence for use of fondaparinux, parenteral DTIs, and DOACs in pediatric patients is more limited and should be reserved for patients that are unable to receive warfarin, UFH, or LMWH.

In conclusion, clinicians now have several anticoagulants in the armamentarium of options for VTE treatment. Consideration of patient preferences and clinical characteristics, along with properties and dosing strategies of each of the agents, is imperative for optimal anticoagulation therapy.

IVC Filter Indications

In select patients who present with acute VTE, the risk of bleeding may be so high that the use of anticoagulation is contraindicated. In such patients, blocking or filtering blood flow from the inferior vena cava (IVC) to prevent pulmonary emboli is considered by inserting an IVC filter. Although this intervention developed over 100 years ago using ligation of the IVC, only in the last 50 years have practical mechanical devices been developed to achieve this with minimal intervention. Many different filters have been designed over the years, and today, retrievable IVC filters are mostly used. There is only one long-term randomized study assessing the efficacy and safety of these filters [73]. In patients with acute VTE randomized to an IVC filter plus anticoagulation compared with those with anticoagulation alone, the long-term

outcomes including death, recurrent VTE, or post-thrombotic syndrome were no different, although those with filters had more DVT compared to those without filters who had more PE. Unfortunately, the study subjects were not the typical patients most likely considered for filter placement, and most of the study patients continued on anticoagulation.

The indications for IVC filter placement, as proposed by professional societies, vary depending on the medical discipline. Examples include the following:

The American College of Chest Physicians advocates for the use of IVC filters as follows:

- *Vena cava filters for the initial treatment of DVT or PE:* For patients with acute proximal DVT or PE, if anticoagulant therapy is not possible because of the risk of bleeding, we recommend placement of an inferior vena cava (IVC) filter (Grade 1C).
- For patients with chronic thromboembolic pulmonary hypertension undergoing pulmonary thromboendarterectomy, we suggest the placement of a permanent vena cava filter before or at the time of the procedure (Grade 2C).

The Society of Interventional Radiology advocates for the uses of IVC filters as follows:

- *Absolute indications (proven VTE):* recurrent VTE (acute or chronic) despite adequate anticoagulation, contraindication to anticoagulation, complication of anticoagulation, and inability to achieve/maintain therapeutic anticoagulation
- *Relative indications (proven VTE):* iliocaval DVT; large, free-floating proximal DVT; difficulty establishing therapeutic anticoagulation; massive PE treated with thrombolysis/thrombectomy; chronic PE treated with thromboendarterectomy; thrombolysis for iliocaval DVT; VTE with limited cardiopulmonary reserve; recurrent PE with filter in place; poor compliance with anticoagulant medications; and high risk of complication of anticoagulation (e.g., ataxia, frequent falls)
- *Prophylactic indications* (no VTE, primary prophylaxis not feasible as a result of high bleeding risk, inability to monitor the patient for VTE, etc.): trauma patients with high risk of VTE, surgical procedure in patient at high risk of VTE, and medical condition with high risk of VTE

One thing that is certain is that filters have been progressively overused. Retrievable filters were developed so that they could be removed once the contraindication against anticoagulation is resolved, as is the case in many patients. Unfortunately, patients with retrievable filters are not always followed up, and in more than 50% patients, such filters are not removed. IVC filters are also associated with serious complications in 1–3% of individuals such as filter migration, filter fracture, perforation of the IVC, and others [74].

Duration of VTE Therapy

One of the more controversial and unsettled aspects of the treatment of VTE is the appropriate duration of treatment [75]. Recurrent VTE is a common problem and occurs in as many as 30% of selected patients over 10 years [76]. The decision to continue treatment requires a continuous benefit/risk analysis, i.e., does the benefit of continuing anticoagulant treatment and preventing recurrent VTE outweigh the risk of bleeding from anticoagulant therapy [77]? The decision is guided by understanding the risk factors for recurrent VTE following an initial episode. The major factors include the following:

- Was the initial episode provoked by a risk factor, such as surgery or trauma, or was it unprovoked (idiopathic)?
- If risk factors were present, were they transient (i.e., reversible) or persistent (such as obesity and cancer)?
- Is the episode a proximal (thigh) DVT or PE or a distal (calf) DVT?
- Are certain biomarkers (D-dimer) positive or negative after several months of therapy?
- Is the patient a male?

Abundant evidence indicates that the risk of recurrent VTE after a provoked VTE with transient risk factors is low enough that only 3 months of therapy is warranted. Patients with a VTE and persistent risk factors or with an idiopathic VTE require longer treatment, and therapy is often recommended to be indefinite with ongoing evaluations for the risk/benefit of such therapy. Patients with a calf vein DVT usually require only 3 months of therapy and, in some cases, only observation and monitoring, but careful surveillance is suggested to look for recurrence, especially if no risk factors are identified. Patients with evidence of residual vein occlusion or an elevated D-dimer after 3–6 months of therapy have a higher risk of recurrence. Persistent risk factors include such things as cancer, a major thrombophilia, or marked obesity. Additionally, males have a higher risk of recurrence than females. Table 17.11 identifies common risk factors and the relative risk for recurrence. Table 17.12 summarizes the recommended duration of therapy and the strength of the recommendation.

Table 17.11 Risk factors for VTE recurrence

Risk factor	Relative risk/hazard ratio (95% CI)
Unprovoked proximal DVT	2.3 (1.8–2.9)
Obesity	1.6 (1.1–2.4)
Male sex	2.8 (1.4–5.7)
Positive D-dimer	2.6 (1.9–3.5)
Residual thrombosis	1.5 (1.1–2.0)
Antiphospholipid antibody	2.4 (1.3–4.1)
Inflammatory bowel disease	2.5 (1.4–4.2)
Hereditary thrombophilia	1.5 (1.1–1.9)

Table 17.12 Duration of anticoagulation in patients with acute DVT of the leg

Type of VTE	Duration of treatment	Recommendation
Provoked isolated distal DVT	3 months	Grade 1B
Unprovoked distal DVT	3 months and then evaluation of risk/benefit ratio of extended therapy	Grade 1B
	For low or moderate bleeding risk → 3 months	Grade 2B
	For high bleeding risk → 3 months	Grade 1B
Provoked proximal DVT	3 months	Grade 1B
Unprovoked proximal DVT	At least 3 months and then evaluation of risk/benefit ratio of extended therapy	Grade 1B
	For low or moderate bleeding risk → extended anticoagulant therapy	Grade 2B
	For high bleeding risk → 3 months	Grade 1B
Second unprovoked DVT	For low bleeding risk → extended anticoagulant therapy	Grade 1B
	For moderate bleeding risk → extended anticoagulant therapy	Grade 2B
	For high bleeding risk → 3 months	Grade 2B
DVT and active cancer	For low or moderate bleeding risk → extended anticoagulant therapy	Grade 1B
	For high bleeding risk → extended anticoagulant therapy	Grade 2B

Notes: Initial and long-term therapy for PE is the same as for proximal DVT. Incidentally found asymptomatic DVT is suggested to be treated with initial and long-term anticoagulation as for comparable patients with symptomatic DVT (Grade 2B)
Abbreviations: *DVT* deep vein thrombosis, *PE* pulmonary embolism

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Antiplatelet Therapy in Cardiovascular Disease

18

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Platelet activation and aggregation plays a maladaptive role in the pathophysiology of ischemic heart disease. Therefore, antiplatelet therapy is essential in the treatment of acute coronary syndromes and stable coronary artery disease. Our understanding of how to apply this therapy has been bolstered by large clinical trials. Drugs that inhibit platelet function predominantly affect key signaling pathways that are required for optimal platelet activation such as the cyclooxygenase-thromboxane pathway (aspirin) and ADP signaling ($P2Y_{12}$ antagonists). The specific agents, dose, and timing of administration vary depending on the cardiovascular indication. Adverse effects of antiplatelet therapy center around bleeding, such as when dual antiplatelet therapy is employed. Given the high prevalence of cardiovascular disease, the hematologist should be comfortable with antiplatelet therapy, balancing the risk of thrombosis versus bleeding based on the clinical context. This chapter will discuss the pharmacology of antiplatelet therapy and the clinical evidence that guides its application in medical practice.

Role of Platelets in the Pathophysiology of Cardiovascular Disease

Ischemic cardiovascular disease, including myocardial infarction, stroke, and peripheral artery disease, is the leading cause of death in most regions of the world. The pathophysiology of cardiovascular disease involves complex interplay between chronic endothelial damage from risk factors such as hyperlipidemia, hypertension, and smoking leading to atherosclerotic plaque formation, and activation of platelets and the coagulation system to create a prothrombotic state.

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Platelets Are Key Mediators in Atherosclerotic Vascular Disease

Platelet adhesion, activation, and aggregation at sites of vascular injury are an essential defense mechanism in normal hemostasis and physiologic thrombosis following trauma. This process is maladaptive in atherothrombosis – clot formation at sites of ruptured or eroded atherosclerotic plaque – which lead to intraluminal thrombosis, vascular occlusion, tissue ischemia, and ultimately necrosis. Disruption of atherosclerotic plaque exposes an injured subendothelium enriched in platelet-adhesive and activating proteins including collagen, other subendothelial matrix, and tissue factor that leads to thrombin formation. Platelets are ideally suited to be activated at sites of high shear stress such as the coronary circulation via initial adhesion to von Willebrand factor and platelet activation by collagen and thrombin which result in granule release and further activation by thromboxane, ADP, and other platelet agonists. Given the essential and primary role of platelets in atherothrombosis, inhibition of platelet function through pharmacologic agents is the cornerstone in management of atherosclerotic vascular disease.

1. *Coronary artery disease (CAD)* involves atherosclerotic narrowing and vascular dysfunction in the coronary arteries and results in ischemic heart disease.
   (a) Stable coronary artery disease manifests as reproducible angina pectoris symptoms or is asymptomatic.
   (b) Acute coronary syndromes (ACS) refer to a spectrum of syndromes from rapidly progressive chest pain (unstable angina) to non-ST segment elevation myocardial infarction (NSTEMI) and ST-segment elevation myocardial infarction (STEMI) where there is unstable atherosclerotic plaque and rapid progression of atherothrombosis.
2. *Cerebrovascular disease* includes both ischemic strokes and transient ischemic attacks. These have multiple etiologies including aortic, carotid, or intracerebral atherosclerosis with atheroembolism, cardiac thromboembolism, and small vessel "lacunar" disease.

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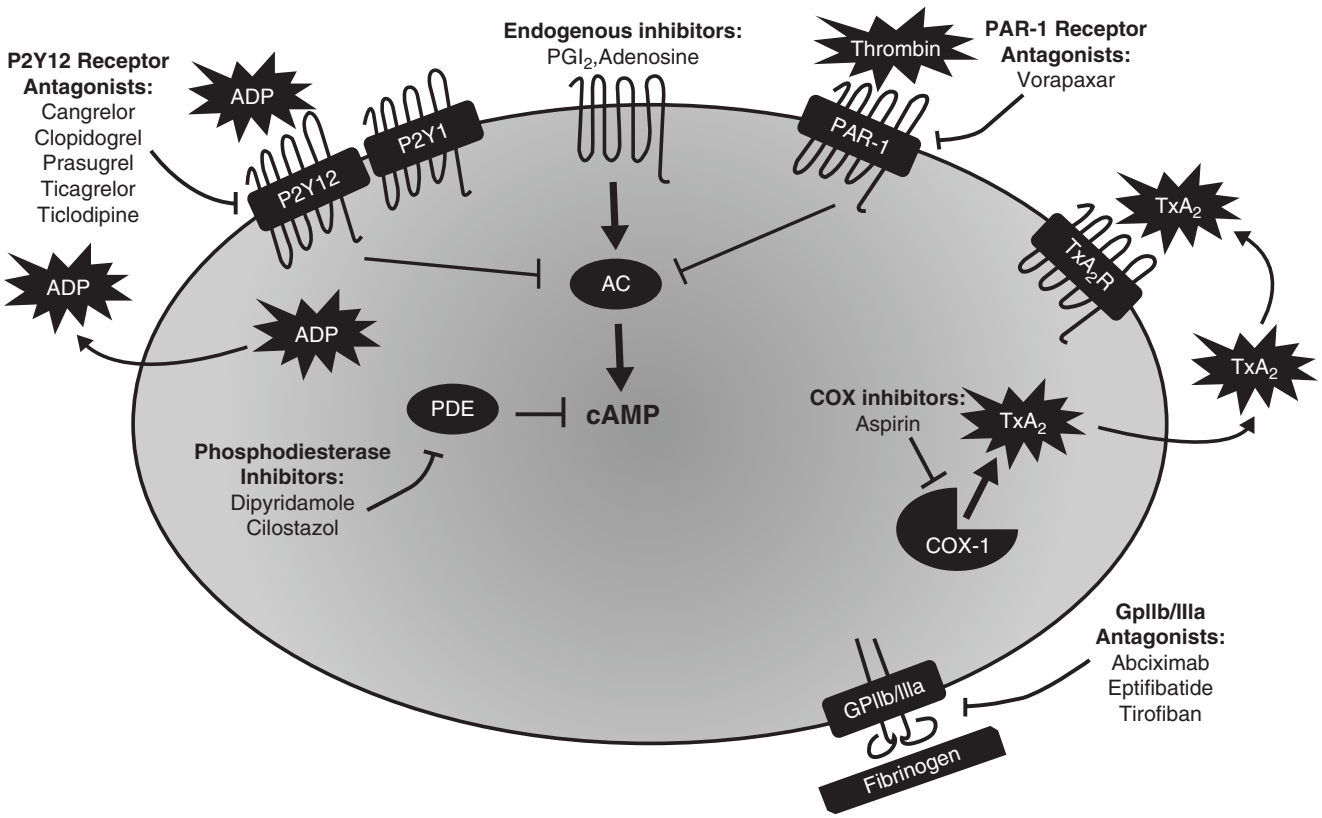

Fig. 18.1 Antiplatelet therapies target signaling pathways required for optimal platelet activation in response to endothelial injury. Platelet activation is mediated by the cyclooxygenase-thromboxane pathway (inhibited by aspirin), ADP signaling (inhibited by $P2Y_{12}$ antagonists), thrombin signaling (inhibited by PAR-1 antagonists), phosphodiesterase (inhibited by cilostazol and dipyridamole). Platelet adhesion and thrombus formation are mediated via the fibrinogen receptor (inhibited by glycoprotein IIb/IIIa antagonists). ADP indicates adenosine diphosphate, COX cyclooxygenase, GP glycoprotein, PGI_2 prostacyclin, PAR protease-activated receptor, TXA_2 thromboxane A_2, TXA_2R thromboxane A_2 receptor

3. *Peripheral artery disease (PAD)* results from atherosclerotic narrowing and vascular dysfunction of the leg, or less commonly the arms, or gut vessels, and as a result of inadequate tissue perfusion can result in stable claudication (buttock, leg, or calf pain with ambulation), acute, or chronic limb ischemia. Mortality in PAD patients largely results from their heightened risk of coronary artery disease and stroke.

Platelets in Venous Thrombosis

The role of platelets in venous thrombosis is less well-defined. The fibrin-rich "red clots" of venous thrombosis are thought to be less dependent on platelet adhesion, activation, and aggregation as an inciting event. However, platelets have a key role in binding to procoagulant endothelium and amplify thrombus formation via positive feedback of the coagulation cascade.

Pharmacologic Targets of Antiplatelet Drugs

Drugs that inhibit platelet function predominantly affect key signaling pathways that are required for optimal platelet activation such as the cyclooxygenase-thromboxane pathway (aspirin), ADP signaling ($P2Y_{12}$ antagonists), thrombin signaling (PAR-1 antagonists), or adhesion such as the fibrinogen receptor integrin $\alpha_{2b}\beta_3$ (glycoprotein IIb/IIIa antagonists) (Fig. 18.1). There is great functional redundancy in platelet activation signaling, and as a result, no single agent completely inhibits platelet function.

Aspirin

Mechanism of Action

Acetylsalicylic acid, later named aspirin, was first prepared in 1853 and gained wide use as an anti-inflammatory over the following century. Aspirin acetylates and irreversibly inhibits cyclooxygenase-1 and cyclooxygenase-2 (COX-1 and COX-2) at a serine residue in the catalytic site, preventing substrate access. COX is the enzyme responsible for prostaglandin H_2 production, the immediate precursor to thromboxane A_2 (TxA_2) and prostaglandins including prostacyclin (PGI_2). In hemostasis and thrombosis, COX-1 and COX-2 have antagonistic functions, with COX-1 leading to

prothrombotic TXA_2 production in the platelet and COX-2 leading to antithrombotic PGI_2 in the endothelium. The antiplatelet properties of aspirin are unique to its usage at low doses, as a 75–150 mg once-daily dose of aspirin is sufficient to inhibit the COX-1 isoform and inhibit platelet production of TXA_2 but not inhibit COX-2 activity in endothelial cells. This difference is due to the inability of anucleated platelets to upregulate COX-1 synthesis, whereas nucleated endothelium can continuously produce additional COX-2 enzyme.

Pharmacokinetics and Pharmacodynamics

Aspirin is rapidly absorbed in the stomach with an oral bioavailability of approximately 40–50% and achieves peak antiplatelet activity within 1 h. Bioavailability and peak plasma levels may be markedly decreased with enteric-coated formulations [1], which require absorption in the higher pH environment of the small intestine. Aspirin has a rapid half-life of 15–20 min, but due to its irreversible inhibition of COX-1 in circulating platelets and megakaryocytes, its effects last for the lifetime of the platelet which is approximately 8–10 days.

Efficacy and Safety

Randomized controlled trials in the late 1980s and early 1990s, such as the Second International Study of Infarct Survival (ISIS) and RISC study which enrolled patients with acute MI [2, 3]; the Swedish Angina Pectoris Aspirin Trial (SAPAT), which included patients with stable CAD [4]; and the Swedish Aspirin Low-dose Trial (SALT), which included patients following stroke [5], demonstrated clear benefit for the use of low-dose aspirin 75–162.5 mg/day for the prevention of cardiovascular events and vascular death. Meta-analysis of secondary prevention trials of low-dose aspirin demonstrates a 15–30% relative reduction in myocardial infarction (MI), stroke, or vascular death in patients with multiple forms of atherosclerotic vascular disease and presenting with an acute coronary syndrome, acute stroke, or a history of MI, stroke, or PAD (6.7% vs 8.2% per year $P < 0.0001$) [6]. For primary prevention of cardiovascular events (e.g., patients who do not have evidence of atherosclerotic vascular disease), low-dose aspirin reduces the incidence of nonfatal MI by approximately 20% (0.18% vs 0.23% per year, $P < 0.0001$, number needed to treat = 2000 patients) but has not been demonstrated to reduce stroke, cardiovascular death, or all-cause mortality [6]. Daily aspirin use might decrease the incidence of and mortality from certain malignancies, namely, colonic adenocarcinoma, a benefit that only becomes apparent after at least 5–10 years of treatment [7, 8], and decreases the recurrence of colonic adenomas [9, 10]. Aspirin compared with placebo reduces venous thromboembolism recurrence (20–40%) [11] but is significantly less effective than systemic anticoagulation.

During aspirin therapy, the relative risk of bleeding is increased by approximately 30–50% and is increased at higher aspirin doses. This correlates to an increase in major gastrointestinal or extracranial bleeding events to 0.1% per year with aspirin versus 0.07% per year with placebo in primary prevention trials, and the risk of bleeding is higher for patients at increased risk for thrombotic vascular events [6]. Aspirin may increase the incidence of hemorrhagic stroke by one to five events per thousand patients per year. Aspirin-related GI toxicity is likely dose-dependent, related to increased COX-1 inhibition in the gastric mucosa. Enteric-coated aspirin does not decrease the risk of GI bleeding. Concomitant acid suppression with proton-pump inhibitors and *helicobacter pylori* eradication are the best strategies for reducing the risk of aspirin-mediated peptic ulcer disease.

Dosing

The relative risk of gastrotoxicity and bleeding increases at higher aspirin dose, but the efficacy of aspirin for prevention of vascular events may be attenuated at doses above 150 mg daily, possibly due to endothelial PGI_2 inhibition. In the largest meta-analysis, comparing aspirin to no-aspirin, doses of 500–1500 mg conferred a 19% relative risk reduction, doses of 160–325 mg conferred a 26% risk reduction, doses of 75–150 mg conferred a 32% risk reduction, and doses <75 mg conferred a 13% risk reduction [12]. The forthcoming ADAPTABLE (Aspirin Dosing: A Patient-centric Trial Assessing Benefits and Long-Term Effectiveness) trial will randomly assign patients with established CAD to a regimen of 81 mg/day or 325 mg/day to help definitively answer the question of optimal aspirin dosing.

Indications

Low-dose aspirin (75–100 mg/day) is indicated for all patients with established cardiovascular disease (CAD, cerebrovascular disease, and PAD) with or without a history of ACS or stroke. On presentation of ACS (unstable angina, NSTEMI, STEMI), a one-time "loading dose" of 162–325 mg aspirin is given for maximal platelet inhibition and to overcome issues with impaired GI absorption. For acute ischemic stroke, patients should be loaded with high-dose (325 mg) aspirin within 48 h of presentation and are discharged on 75–100 mg daily. Aspirin is often given at higher doses (162–325 mg/day) for the 1st year following coronary artery bypass surgery, but evidence supporting this practice is limited. Patients receiving intracoronary stenting during percutaneous coronary intervention (PCI) should receive low-dose aspirin daily indefinitely, due to the risk of stent

thrombosis. Patients with prosthetic heart valves should receive low-dose aspirin therapy alone for a bioprosthetic valve or in addition to anticoagulation with warfarin for a mechanical valve. For primary prevention of atherosclerotic vascular events, patients at low risk (<10% risk of a major atherosclerotic vascular event over 10 years), the benefit of low-dose aspirin is offset by the risk of bleeding. For patients with a 10-year risk of 10–20% without bleeding history or high bleeding risk or all patients with a 10-year risk of >20%, the benefit of low-dose aspirin therapy likely outweighs the bleeding risk. Validated 10-year cardiovascular event calculator tools can be found at: http://tools.acc.org/ASCVD-Risk-Estimator. The United States Preventive Services Task Force gives a grade C recommendation for low-dose aspirin use for the prevention of colon cancer, tailored to the life expectancy and bleeding risk of the patient. Of note, if a patient is taking aspirin for primary prevention of cardiovascular events and needs to start therapeutic anticoagulation (for venous thromboembolism or stroke prevention in atrial fibrillation), it is appropriate to stop aspirin therapy since therapeutic anticoagulation is also protective against atherothrombosis. Refer to Table 18.2 for a summary of indications for aspirin therapy.

Aspirin "Resistance"

Certain patients demonstrate high platelet reactivity despite taking aspirin, and this phenomenon has been weakly linked with an increased risk of vascular events. The lack of optimal platelet inhibition due to aspirin therapy is multifactorial and related to patient characteristics such as body mass index, mean platelet volume, and diabetes or other inflammatory conditions that can increase platelet turnover. Nonsteroidal anti-inflammatory drugs (NSAIDs) *reversibly* inhibit platelet COX-1 and therefore may decrease the efficacy of aspirin due to its short half-life. Celecoxib and diclofenac are NSAIDs that do not interfere with the antiplatelet action of aspirin. The term aspirin "resistance" is misleading and is more accurately represented as the inter-patient variability in aspirin response. Patients with a high platelet turnover state such as diabetes, obesity, essential thrombocythemia, and post-CABG may have more persistent platelet COX-1 inhibition with twice daily dosing. However, no strategy of aspirin dose adjustment in patients at risk for, or demonstrated to have, a diminished response to aspirin therapy has resulted in improved cardiovascular outcomes to date.

P_2Y_{12} Antagonists and Dual Antiplatelet Therapy

Mechanism of Action

Platelet activation via autocrine and paracrine signaling through the G protein-coupled ADP receptors $P2Y_1$ and $P2Y_{12}$ is required for maximal platelet activation and propagation of thrombus. The ADP receptor $P2Y_{12}$ is the drug target of the thienopyridines (ticlopidine, clopidogrel, and prasugrel) and non-thienopyridines (ticagrelor and cangrelor). These agents effectively block ADP-induced platelet aggregation and induce submaximal aggregation in response to non-ADP agonists (compared in Table 18.1). Due to the high thrombotic risk despite aspirin therapy in patients receiving intracoronary stents or suffering acute coronary syndromes, the concept of dual antiplatelet therapy (DAPT) with both aspirin and a $P2Y_{12}$ inhibitor emerged as the optimal strategy to lower ischemic events in these patients (Table 18.2).

Ticlopidine

The first $P2Y_{12}$ antagonist approved by the FDA in 1991, ticlopidine, helped usher in the concept of DAPT by demon-

Table 18.1 Pharmacological properties of the $P2Y_{12}$ inhibitors

	Clopidogrel	Prasugrel	Ticagrelor	Cangrelor
Chemical class	Thienopyridine	Thienopyridine	Cyclopentyl-triazolopyrimidine	Stabilized ATP analogue
Administration	Oral	Oral	Oral	Intravenous
Dose	300–600 mg load, then 75 mg daily	60 mg load, then 10 mg daily	180 mg load, then 90 mg twice daily	30 μg/kg bolus, then 4 μg/kg/min infusion
Prodrug	Yes	Yes	No	No
Onset of effect	2–8 h	30 min to 4 h	30 min to 4 h	Immediate
Duration of effect	3–10 days	7–10 days	3–5 days	30–60 min
Half-life of active metabolite	~30 min	~30–60 min	~7–9 h	3–6 min
Response to platelet transfusion	Potentially	Potentially	Unlikely	Not applicable
Influenced by genetic variation	Yes	No	No	No
Interactions with CYP-targeted drugs or P-glycoprotein	CYP2C19, P-glycoprotein	None	CYP3A4, CYP3A5 P-glycoprotein	None
Approved indications	ACS and PCI in stable CAD	ACS undergoing PCI	ACS	PCI in patients with ACS and stable CAD

Table 18.2 Indications for aspirin therapy

Indication for aspirin	Dose	Comments
Stable coronary artery disease (no history of ACS)	75–100 mg/day	
Stable coronary artery disease (history of MI with or without PCI)	75–100 mg/day	
ACS (unstable angina, NSTEMI, STEMI: acute presentation)	162–325 mg once, followed by 75–100 mg/day	
Acute stroke or TIA	162–325 mg once or for duration of admission, then 75–100 mg daily	Consider DAPT for 90 days in patients with 70–99% stenosis of intracranial artery
CABG surgery	162–325 mg/day for 1 year, then 75–100 mg/day	
History of PCI	75–100 mg/day	
Peripheral arterial disease	75–100 mg/day	
History of stroke	75–100 mg/day	
Prosthetic heart valve	75–100 mg/day	
Primary prevention of atherosclerotic vascular disease, high-risk patient (10-year risk of a major atherosclerotic vascular event >20%)	75–100 mg/day	Reasonable in patients with 10-year risk of 10–20% without bleeding history or high bleeding risk

ACS acute coronary syndrome, *DAPT* dual antiplatelet therapy, *MI* myocardial infarction, *PCI* percutaneous coronary intervention, *NSTEMI* non-ST-elevation myocardial infarction, *STEMI* ST-elevation myocardial infarction, *CABG* coronary artery bypass graft

strating the superiority of aspirin plus ticlopidine compared with aspirin alone or aspirin plus warfarin in preventing thrombotic complications following coronary artery stent placement. Ticlopidine was associated with bone marrow suppression, including neutropenia, thrombocytopenia, and aplastic anemia in addition to thrombotic thrombocytopenic purpura. These adverse effects, although rare (0.5–1%), and the development of clopidogrel with an improved safety profile have resulted in removal of ticlopidine from clinical use.

Clopidogrel

Pharmacokinetics and Pharmacodynamics

Clopidogrel is rapidly absorbed, and 85% of the drug is hydrolyzed by esterases into an inactive carboxylic acid. The remaining 15% of clopidogrel undergoes two-step metabolism by cytochrome P450 enzymes, namely, CYP2C19, to generate a labile metabolite that irreversibly inhibits $P2Y_{12}$ on platelets as they pass through the liver. As a result of this required metabolism, clopidogrel has a delayed onset of action, and a loading dose of 300 mg does not result in maximal platelet inhibition for at least 6 h. A 600 mg loading dose results in maximal platelet inhibition within 2–4 h. Daily dosing of clopidogrel at 75 mg is appropriate since it inhibits $P2Y_{12}$ for the lifetime of the platelet.

Efficacy and Safety

The efficacy of clopidogrel was demonstrated in the Clopidogrel vs Aspirin in Patients at risk of Ischemic Events (CAPRIE) trial [13] published in 1996, which included 19,185 patients who had a history of either MI, stroke, or symptomatic PAD. Clopidogrel compared with aspirin therapy reduced the primary endpoint of stroke, MI, or vascular death by 8.7% (absolute risk reduction 0.51%, $P = 0.04$) per year with no difference in hemorrhagic events. However, because of the greater cost of clopidogrel, aspirin remained more commonly used for secondary prevention.

Clopidogrel has gained widespread use as part of DAPT along with aspirin in patients who are high risk for thrombosis. The Clopidogrel in Unstable Angina to Prevent Recurrent Events (CURE) trial [14], published in 2001, randomly assigned 12,562 patients with acute coronary syndrome who presented within 24 h to receive clopidogrel (300 mg loading dose, then 75 mg daily) or placebo in addition to aspirin (75–325 mg daily). After an average of 9 months, there was a 20% relative reduction (9.3% vs 11.4%, $P < 0.001$) in the primary endpoint of cardiovascular death, nonfatal MI, or stroke. This benefit was especially great in the subset of patients who received percutaneous coronary intervention but also present among patients who underwent coronary artery bypass grafting (CABG) or received no intervention. Clopidogrel-treated patients experienced more major bleeding than placebo (3.7% vs 2.7%, $P = 0.001$). DAPT with clopidogrel reduced adverse ischemic outcomes in ST-elevation MI in the Clopidogrel and Metoprolol in Myocardial Infarction Trial (COMMIT) trial [15] and in acute MI patients receiving fibrinolytic therapy in the Clopidogrel as Adjunctive Reperfusion Therapy (CLARITY-TIMI 28) study [16] as well as in patients with stable angina undergoing elective coronary stenting in the Clopidogrel for the Reduction of Events During Observation (CREDO) trial [17]. In all of these studies, which enrolled patients with ACS or post-stenting, DAPT provided a net clinical benefit despite an increased risk of bleeding.

The concept of DAPT was logically next tested in secondary prevention in non-ACS patients and primary prevention in high-risk patients. In the Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management, and Avoidance (CHARISMA) trial [18], published in 2006, 15,603 patients at increased risk for vascular events did not experience overall benefit from clopidogrel plus aspirin versus aspirin plus placebo, but the subgroup of secondary prevention patients (12,153 patients with established CAD, cerebrovascular disease, or PAD) derived a benefit from DAPT that was statistically significant. Moreover, the subset of "CAPRIE-like"

patients with a history of MI, stroke, or symptomatic PAD experienced a reduction in cardiovascular death, MI, or stroke (7.3% vs 8.8%, $P = 0.01$ over 30 months) [19].

In cerebrovascular disease, the Management of Atherothrombosis With Clopidogrel in High-Risk Patients (MATCH) trial [20], published in 2004, investigated DAPT in patients with a history of stroke within 3 months and other vascular risk factors and failed to show a benefit with the addition of aspirin to clopidogrel versus clopidogrel monotherapy alone. There is some evidence based on two small trials that treatment with DAPT within 24 h of acute minor stroke or TIA and continued for 3 months reduces the incidence of subsequent ischemic stroke [21, 22]. Additionally, guidelines suggest that 3 months of DAPT may be reasonable within 30 days of a stroke due to 70–99% stenosis of a major intracranial artery.

Clopidogrel Resistance

Due to the more variable bioavailability and dependence on hepatic bioactivation, as many as one-third of patients demonstrate a lack of expected platelet inhibition despite clopidogrel therapy – termed high on-treatment platelet reactivity. This scenario is associated with an increased risk of thrombotic events. Observational studies suggested proton-pump inhibitors (PPIs), which inhibit CYP450 enzymes to varying degrees, may be associated with a decreased platelet response to clopidogrel. However, in the large randomized Clopidogrel and the Optimization of Gastrointestinal Events Trial (COGENT) [23], patients with an indication for DAPT with aspirin plus clopidogrel were given the PPI omeprazole or placebo. There was a 60% relative reduction in GI events in the omeprazole-treated patient population and importantly no difference in thrombotic vascular outcomes. Loss-of-function polymorphisms in CYP2C19 result in decreased bioactivation of clopidogrel and higher on-treatment platelet reactivity. Clopidogrel treatment in patients with one or two loss-of-function CYP2C19 alleles may be associated with an increased risk of thrombotic events, but studies have produced conflicting results [24–26]. Three large prospective randomized clinical trials have attempted to address high on-treatment platelet reactivity or the presence of loss-of-function CYP2C19 alleles with a strategy of double-dose clopidogrel. While this strategy may result in lower on-treatment platelet reactivity, it did not demonstrate improved cardiovascular outcomes. These results do not support the practice of modifying clopidogrel therapy based on on-treatment platelet reactivity.

Prasugrel

Pharmacokinetics and Pharmacodynamics

Prasugrel is rapidly absorbed and undergoes activation in a single step by CYP450 enzymes into an active metabolite with a ~4 h half-life that irreversibly inhibits the $P2Y_{12}$ receptor. Prasugrel is much less dependent on CYP2C19 for activation, and there is no evidence that CYP polymorphisms affect its efficacy.

Efficacy and Safety

Prasugrel results in more potent inhibition of ADP-induced platelet activation compared with clopidogrel. The efficacy of prasugrel was demonstrated in the Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel (TRITON-TIMI 38) trial [27], published in 2007, in which 13,608 patients presenting with acute coronary syndrome undergoing PCI were randomized to loading and maintenance with either clopidogrel or prasugrel (60 mg load, 10 mg daily maintenance) following angiography at the time of PCI. There was a significant reduction in the combination of nonfatal MI, nonfatal stroke, and cardiovascular death at 15 months in favor of prasugrel over clopidogrel (event rate of 9.9% vs 12.1%, $P < 0.001$). The benefit was particularly notable in patients with STEMI, diabetes, and those experiencing recurrent events. There was a small but statistically significant increase in fatal bleeding that was particularly apparent in patients over the age of 75, with a weight less than 60 kg, or with a history of stroke or TIA. Prasugrel is contraindicated in patients with a history of stroke or TIA, due to an increased risk of intracranial hemorrhage. In a population of patients with non-ST-elevation MI who did not undergo revascularization, in the Targeted Platelet Inhibition to Clarify the Optimal Strategy to Medically Manage Acute Coronary Syndromes (TRILOGY-ACS) trial [28], there was no overall benefit to DAPT with prasugrel compared with clopidogrel. However, in a prespecified subgroup analysis, in those patients undergoing coronary angiography before randomization, prasugrel was superior to clopidogrel. This may represent a subgroup of ACS patients more likely to be experiencing acute plaque rupture, where more intensive antiplatelet therapy is beneficial. Despite these findings, prasugrel is currently indicated only for patients undergoing PCI in the setting of ACS.

Ticagrelor

Mechanism of Action, Pharmacokinetics, and Pharmacodynamics

Ticagrelor is an oral cyclopentyl-triazolopyrimidine that does not require metabolic activation. It is a reversible inhibitor of $P2Y_{12}$ that does not interact with the ADP-binding site of the receptor as do thienopyridines but instead inhibits $P2Y_{12}$ via allosteric modulation. An active metabolite generated by CYP3A4 is responsible for 30–40% of the antiplatelet effect of the drug, and therefore administration with potent CYP3A4 inducers (such as certain anticonvulsants, barbiturates, rifampin, and certain non-nucleoside reverse transcriptase inhibitors) or inhibitors (such as azole antifungals and protease inhibitors) should be avoided. Ticagrelor

has a plasma half-life of 8–12 h and requires twice-daily dosing. Ticagrelor has a faster onset and offset, plus a more potent and reliable antiplatelet effect compared with clopidogrel. Ticagrelor has off-target effects, namely, inhibition of the equilibrate nucleoside transporter-1 (ENT-1), resulting in increased levels of adenosine. Beneficial effects of ENT-1 inhibition include increased platelet inhibition (via the adenosine A_{2a} receptor), increased arteriolar vasodilation, reduction in inflammation, and decreased myocardial necrosis following injury [29, 30]. Stimulation of pulmonary vagal C fibers via increased adenosine likely contributes to the subjective dyspnea experienced by ~10% of patients while taking ticagrelor.

Efficacy and Safety

The Platelet Inhibition and Patient Outcomes (PLATO) trial, [31], published in 2009, randomized 18,624 patients with ACS to receive ticagrelor (180 mg loading dose following by 90 mg twice daily) or clopidogrel loading and maintenance in addition to aspirin in both groups for 12 months. Many patients had already been pretreated with clopidogrel, and ticagrelor could be administered before or after angiography, but before PCI. Ticagrelor treatment reduced the composite endpoint of death from vascular causes, MI, or stroke compared to clopidogrel (9.8% vs 11.7%, $P < 0.001$), driven by a 21% relative reduction in cardiovascular death and a 16% reduction in MI. This benefit was achieved in patients who ultimately received revascularization with PCI or CABG or received medical management alone. Ticagrelor was associated with improved outcomes among a wide range of subgroups including those with recurrent events, diabetes, chronic kidney disease, age >75 years old, weight <60 kg, or history of TIA or stroke, and the benefit was irrespective of loss-of-function CYP2C19 polymorphisms. There was no difference in total major bleeding between ticagrelor- and clopidogrel-treated patients (11.6% vs 11.2%) or life-threatening or fatal bleeding (5.8% in both treatment arms), though non-CABG major bleeding was significantly increased with ticagrelor.

In patients with stable CAD but a history of MI within the previous 1–3 years (83% had previous PCI), and other high-risk features, extended DAPT with aspirin plus ticagrelor at a dose of 90 mg or 60 mg twice daily was compared with aspirin plus placebo in 21,162 patients in the Prevention of Cardiovascular Events in Patients with Prior Heart Attack Using Ticagrelor Compared to Placebo on a Background of Aspirin (PEGASUS-TIMI 54) trial published in 2015 [32]. Notably, patients with a history of ischemic stroke were excluded. After 36 months, the combined endpoint of cardiovascular death, nonfatal MI, or stroke was reduced in both arms by approximately 15% (7.85% for ticagrelor 90 mg and 7.77% for ticagrelor 60 mg vs 9.04% for placebo, $P = 0.008$ and $P = 0.004$, respectively), driven by the reduction in MI and stroke. Major bleeding was increased in both the ticagrelor 90 mg group (2.6% vs 1.06%) and the ticagrelor 60 mg group (2.3% vs 1.06%) compared with placebo. Based on the results of PEGASUS, ticagrelor at 60 mg twice daily was approved for use in addition to low-dose aspirin in patients with a history of MI beyond the 1st year. In addition to dyspnea, the incidence of gout and ventricular pauses is slightly increased in patients receiving ticagrelor.

Patients with PAD had increased risk of ischemic events and benefitted from DAPT with ticagrelor in the PLATO and PEGASUS trials. In contrast, there was no difference in cardiovascular death, MI, or ischemic stroke with ticagrelor monotherapy compared with clopidogrel monotherapy when assessed in 13,885 patients with symptomatic PAD in the Examining Use of Ticagrelor in Peripheral Artery Disease (EUCLID) trial [33]. A benefit for ticagrelor in the treatment of stroke has also not been demonstrated. In 13,199 patients with an acute non-severe stroke or a high-risk transient ischemic attack who did not receive thrombolysis, ticagrelor did not significantly reduce the incidence of stroke, MI, or cardiovascular death compared with aspirin in the Acute Stroke or Transient Ischaemic Attack Treated with Aspirin or Ticagrelor and Patient Outcomes (SOCRATES) trial [34].

Cangrelor

Despite the refinement in $P2Y_{12}$ inhibition via prasugrel and ticagrelor, these drugs still have the limitations inherent in any oral antiplatelet agent including variable absorption (which could be exacerbated by emesis, cardiogenic shock, or therapeutic hypothermia), delayed onset of platelet inhibition, and a long offset of action.

Pharmacokinetics/Pharmacodynamics

Cangrelor is an intravenous ADP analogue that does not require metabolic activation, reversibly binds to the $P2Y_{12}$ receptor, and achieves >80% platelet inhibition within minutes. The very short half-life of 3–6 min, which is not influenced by age, genetics, or renal function, means that platelet function returns to normal within 60 min of stopping cangrelor infusion.

Efficacy and Use

The Cangrelor versus Standard Therapy to Achieve Optimal Management of Platelet Inhibition (CHAMPION PHOENIX) trial [35], published in 2013, assessed whether cangrelor versus clopidogrel at the time of PCI resulted in reduced thrombotic complications. The study randomized 11,145 patients undergoing PCI for stable angina or ACS to receive either cangrelor (30 μg/kg bolus followed by infusion at 4 μg/kg/min for completion of PCI or at least 2 h) or clopidogrel (300 mg or 600 mg loading dose) at the time of PCI. Patients receiving cangrelor received a 600 mg clopidogrel loading dose at the end of the procedure. The combined endpoint of death, MI, ischemia-driven revascularization, or stent thrombosis at 48 h was significantly reduced in the cangrelor-treated group (4.7%

vs 5.9%, $P = 0.005$), primarily driven by a reduction in MI and stent thrombosis, and these benefits were maintained at 30 days. There was no significant increase in severe bleeding or transfusions in patients receiving cangrelor. Meta-analysis including the CHAMPION PHOENIX trial plus the two other large-scale randomized cangrelor trials, CHAMPION PCI and CHAMPION PLATFORM (24,910 patients in total), demonstrated consistent reductions in MI and stent thrombosis at 48 h and 30 days and across all major subgroups [36]. The FDA approved cangrelor in 2015 for use as an adjunct to PCI in patients who have not received pre-treatment with a $P2Y_{12}$ antagonist and who are not receiving glycoprotein IIb/IIIa inhibitors. Cangrelor also has a theoretical role as a bridging agent in high-risk patients awaiting CABG or emergent noncardiac surgery who require $P2Y_{12}$ inhibition after oral ADP antagonist discontinuation. The feasibility of this strategy has been demonstrated for CABG in the phase II BRIDGE trial published in 2012 [37]. Treatment with cangrelor requires transition to an oral $P2Y_{12}$ inhibitor. Clopidogrel 600 mg or prasugrel 60 mg can be administered immediately following discontinuation of cangrelor infusion. Ticagrelor 180 mg can be administered anytime during cangrelor infusion or immediately upon cangrelor discontinuation.

Dual Antiplatelet Therapy (DAPT)

Duration

DAPT with aspirin and a $P2Y_{12}$ antagonist is an essential component of medical therapy to reduce ischemic events in acute coronary syndromes and stent thrombosis in those undergoing coronary stenting, but these benefits come at the cost of increased bleeding risk. Determining the duration of DAPT represents a challenge and has clinical equipoise.

Several trials have examined a potential benefit for extending DAPT beyond 12 months in high-risk patients (post-PCI or history of MI, often both). Meta-analyses of trial data including over 30,000 stented patients (not including high-risk ACS) demonstrate that extended DAPT results in a reduced rate of stent thrombosis, MI, or stroke but increased bleeding and increased overall mortality, driven by noncardiovascular death [38]. However, among patients with a history of ACS, extended DAPT reduces ischemic events and cardiovascular death with no effect on overall mortality [39]. The decision to extend DAPT beyond 12 months is influenced by patient-specific risk factors (e.g., history of MI, smoking, diabetes), clinical presentation at the time of PCI (ACS being higher risk than non-ACS), and procedural issues such as longer, narrower or bifurcation stents that pose increased thrombotic risk. Increased thrombotic risk needs to be weighed against factors that increase the likelihood of bleeding while on DAPT such as concurrent anticoagulation, advanced age, low body weight, female sex, anemia, and chronic steroid or NSAID use.

Conversely, the use of second-generation drug-eluting stents has reduced the risk of late stent thrombosis. Several studies have examined shorter duration DAPT of 6 or even 3 months following PCI, and this strategy appears reasonable in patients with stable CAD. Furthermore, large trials are underway studying the safety and efficacy of single antiplatelet therapy with ticagrelor after only 1 month of DAPT (GLOBAL LEADERS trial) and after 3 months of DAPT in higher-risk patients (TWILIGHT trial).

Current guidelines (Fig. 18.2) state that for patients with stable CAD and no history of PCI, DAPT is not recommended. For patients with stable CAD undergoing elective PCI, DAPT should be administered for at least 1 month following bare-metal stent (BMS) placement, and a minimum of 6 months for drug-eluting stent (DES) placement. In patients with stable CAD who develop a high risk of bleeding or have a bleeding complication, discontinuation of the $P2Y_{12}$ antagonist after 3 months is reasonable. Patients with ACS are at a higher risk for recurrent thrombotic events. Patients presenting with ACS treated with BMS or DES should receive DAPT for at least 12 months, and prolonging DAPT beyond 12 months may be reasonable. In patients with ACS who develop a high risk of bleeding or have a bleeding complication, discontinuation of the $P2Y_{12}$ antagonist after 6 months is reasonable.

Use with Anticoagulation

Therapeutic oral anticoagulation is indicated for venous thromboembolism or systemic embolism prophylaxis in atrial fibrillation. DAPT in combination with oral anticoagulation, so-called triple therapy, is associated with a three- to fourfold higher risk of bleeding complications. Only clopidogrel is currently recommended for use in DAPT in patients receiving anticoagulation, given the higher bleeding risk associated with prasugrel and ticagrelor. Evidence from two trials (What Is the Optimal Antiplatelet and Anticoagulant Therapy in Patients with Oral Anticoagulation and Coronary Stenting [WOEST] and Open-Label, Randomized, Controlled, Multicenter Study Exploring Two Treatment Strategies of Rivaroxaban and a Dose-Adjusted Oral Vitamin K Antagonist Treatment Strategy in Subjects with Atrial Fibrillation who Undergo Percutaneous Coronary Intervention [PIONEER AF-PCI] studies) suggest in patients receiving anticoagulation for stroke prevention in atrial fibrillation who undergo PCI and are at an increased risk of bleeding, it is safe to use $P2Y_{12}$ inhibitor monotherapy, rather than DAPT [40, 41]. Other large trials are underway to address optimal antithrombotic therapy in patients with atrial fibrillation undergoing PCI.

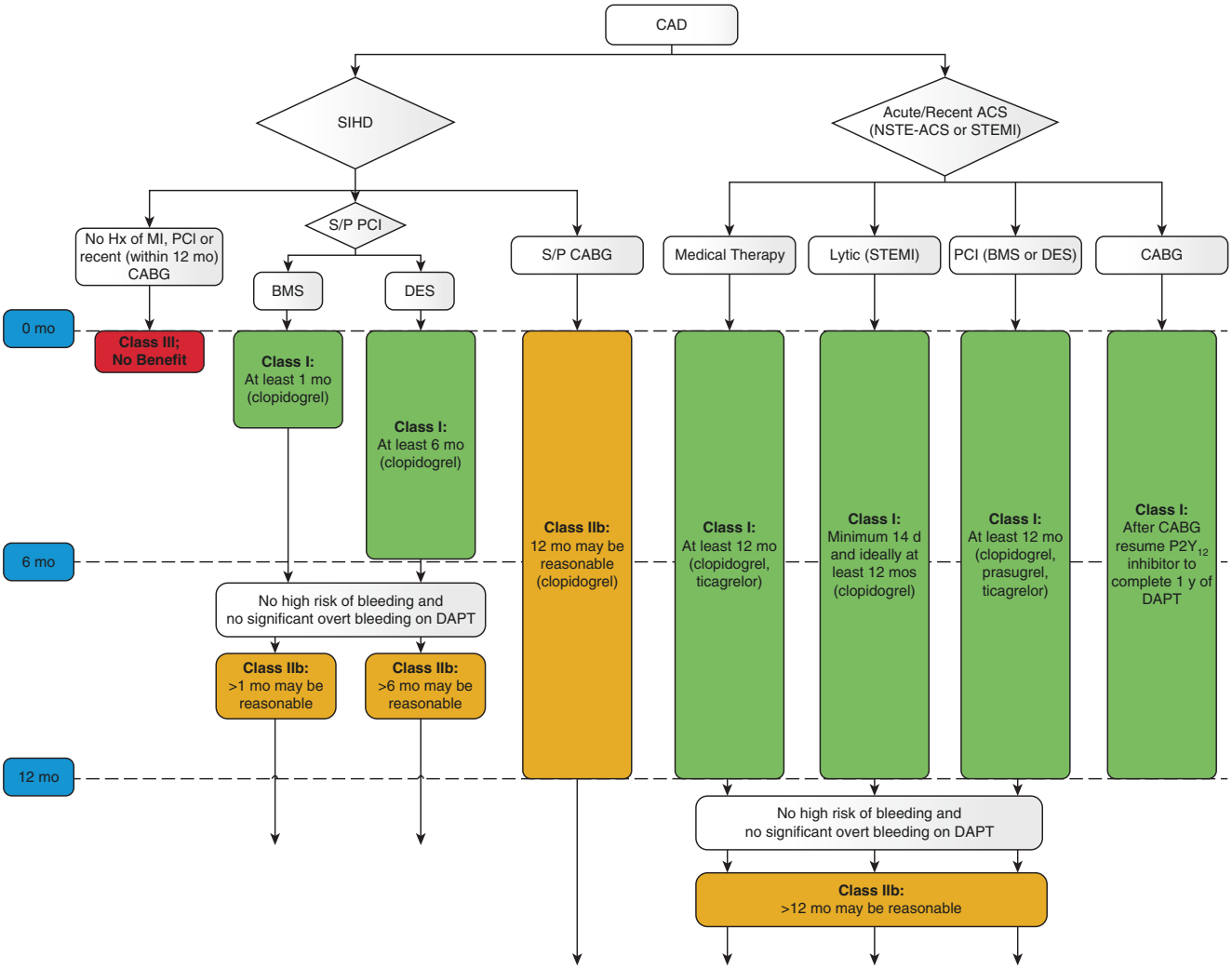

Fig. 18.2 Master treatment algorithm for duration of $P2Y_{12}$ inhibitor therapy in patients with CAD treated with DAPT. Colors correspond to Class of Recommendation in Table 18.1. Clopidogrel is the only currently used $P2Y_{12}$ inhibitor studied in patients with SIHD undergoing PCI. Aspirin therapy is almost always continued indefinitely in patients with CAD. Patients with a history of ACS >1 year prior who have since remained free of recurrent ACS are considered to have transitioned to SIHD. For patients treated with DAPT after DES implantation who develop a high risk of bleeding (e.g., treatment with oral anticoagulant therapy or those undergoing major surgery), or who develop significant overt bleeding, discontinuation of $P2Y_{12}$ inhibitor therapy after 3 months for SIHD or after 6 months for ACS may be reasonable. Arrows at the bottom of the figure denote that the optimal duration of prolonged DAPT is not established. ACS indicates acute coronary syndrome, BMS bare-metal stent, CABG coronary artery bypass graft surgery, CAD coronary artery disease, DAPT dual antiplatelet therapy, DES drug-eluting stent, Hx history, lytic, fibrinolytic therapy, NSTE-ACS non-ST-elevation acute coronary syndrome, PCI percutaneous coronary intervention, SIHD stable ischemic heart disease, S/P status post, and STEMI ST-elevation myocardial infarction

Glycoprotein IIb/IIIa Inhibitors

Mechanism of Action, Pharmacokinetics and Pharacodynamics

Three approved intravenous agents target the integrin-type fibrinogen receptor glycoprotein IIb/IIIa (GPIIb/IIIa) ($\alpha_{2b}\beta_3$ integrins), which is required for fibrin cross-linking and platelet aggregation. Abciximab is a humanized version of a murine antibody directed against GPIIb/IIIa, which at the recommended bolus dose plus 12-h infusion will block >80% of receptors and reduce ADP-induced platelet aggregation to <20% of baseline within 2 h. After discontinuation of abciximab, recovery of platelet function is slow, over 24–72 h. Tirofiban is a non-peptide tyrosine derivative that selectively binds GPIIb/IIIa with a half-life of 1.5–2 h and is partially renally cleared. Eptifibatide is a synthetic cyclic heptapeptide with high affinity for the GPIIb/IIIa active site. It is renally cleared and the duration of platelet inhibition is prolonged in patients with renal impairment receiving eptifibatide. For

both tirofiban and eptifibatide, inhibition of platelet function occurs within minutes and recovers above 50% of baseline within 4 h after stopping infusion. All three GPIIb/IIIa inhibitors have been associated with thrombocytopenia, although the highest risk is with abciximab, at a frequency of 1–2%.

Efficacy and Use

Several trials demonstrated net clinical benefit for GPIIb/IIIa inhibitors despite increased bleeding risk in high-risk ACS patients undergoing PCI, especially when used procedurally to reduce the thrombotic complications of PCI. However, there is no role for routine use of GPIIb/IIIa antagonists in the current era of highly effective $P2Y_{12}$ inhibition. However, on a provisional basis, GPIb/IIIa inhibitors are appropriate in select high-risk PCI scenarios or during thrombotic complications of PCI.

Protease-Activated Receptor 1 (PAR-1) Antagonists

Vorapaxar

Mechanism of Action, Pharmacokinetics, and Pharmacodynamics

PAR-1 is a G protein-coupled thrombin receptor that is responsible for the majority of thrombin-induced platelet activation. Human platelets express two thrombin-activated receptors, PAR-1 and PAR-4. Vorapaxar is a selective PAR-1 antagonist that blocks thrombin-mediated platelet activation. Vorapaxar is rapidly absorbed, has high bioavailability, a long half-life of ~310 h, and is metabolized by hepatic CYP3A4.

Efficacy and Safety

In the Phase III Thrombin Receptor Antagonist for Clinical Event Reduction in Acute Coronary Syndrome (TRACER) trial investigating the addition of vorapaxar (40 mg loading dose plus 2.5 mg maintenance) to standard antiplatelet therapy (aspirin plus clopidogrel in >90% of patients) in non-ST-elevation ACS, the increase in moderate and severe bleeding, especially intracranial bleeding (1.1% vs 0.2%, $P < 0.001$), resulted in early discontinuation of the trial [42]. The Thrombin Receptor Antagonist in Secondary Prevention of Atherothrombotic Ischemic Events (TRA 2P-TIMI 50) trial evaluated the role of vorapaxar in secondary prevention of ischemic events in 26,449 patients with either a history of MI, PAD, or ischemic stroke. Patients received either vorapaxar 2.5 mg or placebo in addition to optimal medical therapy, including DAPT in 58% of patients. After 30 months, there was a significant reduction in the combined endpoint of cardiovascular death, MI, or stroke (9.3% vs 10.5%, $P < 0.001$) mainly driven by a reduction in MI [43]. This benefit came with an approximately twofold increase in moderate or severe bleeding, including intracranial bleeds. Patients with a history of stroke demonstrated increased bleeding and intracranial hemorrhage without evidence of reduction in ischemic events. Patients entering the study based on a history of MI demonstrated a significant reduction in the combined endpoint without a significant increase in intracranial and fatal bleeding. Additionally, patients enrolled based on a history of PAD demonstrated a significant reduction in acute limb ischemia events and peripheral revascularizations suggesting a potential use for vorapaxar in PAD to prevent major adverse limb events [44]. Vorapaxar was approved for clinical use by the FDA in 2014 for secondary prevention of cardiovascular events in patients with a history of MI or PAD. It is contraindicated in patients with a history of stroke, transient ischemic attack, or intracranial hemorrhage.

Dipyridamole

Mechanism of Action, Pharmacokinetics, and Pharmacodynamics

Dipyridamole is a pyrimidopyrimidine derivative that has vasodilator and antiplatelet properties that are likely related to phosphodiesterase inhibition and blockade of adenosine uptake, leading to increased signaling via A_2 receptors and increased intraplatelet cyclic AMP, which inhibits platelet activation. Dipyridamole is marketed in combination with aspirin as a delayed-release formation to improve its bioavailability and has a half-life of ~10 h.

Efficacy and Safety

The combination of extended-release dipyridamole 200 mg with aspirin 25 mg twice daily has a role in the secondary prevention of stroke or transient ischemic attack based on the European Stroke Prevention Study (ESPS-2) and Aspirin Plus Dipyridamole Versus Aspirin Alone After Cerebral Ischaemia of Arterial Origin (ESPRIT) trials which showed an ~20% relative reduction in vascular events compared with aspirin alone, although the aspirin dose was often quite low ($\leq$ 50 mg in 50% of patients) [45, 46]. However, in the Prevention Regimen for Effectively Avoiding Second Strokes (PRoFESS) trial, the combination of dipyridamole 200 mg and aspirin 25 mg twice daily compared with clopidogrel 75 mg daily failed to reach predefined criteria for noninferiority, and there were more major bleeding events, including intracranial bleeding, in the group receiving dipyridamole plus aspirin [47]. Meta-analyses support the superiority of

the combination of dipyridamole plus aspirin versus aspirin alone for prevention of vascular events in patients with a history of stroke or transient ischemic attack but suggest no evidence of superiority in patients with a history of CAD or PAD. Headache is common adverse effect of dipyridamole and results in a discontinuation of the medication in about 10% of patients.

Cilostazol

Mechanism of Action, Pharmacokinetics, and Pharmacodynamics

Cilostazol is a 2-oxoquinoline derivative that acts as a phosphodiesterase-3 inhibitor to increase intracellular cyclic AMP. As a result, it also has vasodilatory properties and may decrease smooth muscle cell proliferation after arterial injury. It is metabolized by CYP450 enzymes, has a half-life of about 11 h, and is excreted by the kidneys.

Efficacy and Safety

Meta-analysis of several small trials has suggested that cilostazol 50 mg or 100 mg twice daily improves pain-free walking distance (claudication symptoms) in patients with PAD [48], and it is approved for this indication only. Small- and medium-sized trials of patients with recent stroke have suggested that cilostazol is non-inferior to aspirin for stroke secondary prevention, with no increase in bleeding risk. After cardiac stenting, addition of cilostazol to standard DAPT further decreases platelet reactivity, and numerous small trials have demonstrated a reduction in cardiovascular events including in-stent restenosis and stent thrombosis with no significant increase in major bleeding, though any potential benefit really needs to be demonstrated in appropriately powered trials [49]. Due to the potentially hazardous cardiac effects of phosphodiesterase-3 inhibition, cilostazol is contraindicated in patients with a history of heart failure or left ventricular ejection fraction <40%. Cilostazol is associated with headache, diarrhea, and dizziness that frequently results in drug discontinuation.

Conclusion

Given the fundamental role of platelet activation and aggregation in atherothrombosis, antiplatelet therapy is an essential element in managing ischemic heart disease. Aspirin remains the backbone of antiplatelet therapy for both ACS and stable CAD. A $P2Y_{12}$ antagonist is used in addition to aspirin when DAPT is indicated. Dual antiplatelet therapy is recommended for at least 1 year following acute coronary syndromes regardless of whether treatment includes coronary intervention (PCI or CABG) or not. For patients with stable CAD undergoing elective PCI, DAPT should be administered for at least 1 month following bare-metal stent placement and a minimum of 6 months for drug-eluting stent placement. Patients presenting with ACS treated with BMS or DES should receive DAPT for at least 12 months, and prolonging DAPT beyond 12 months may be reasonable in patients at high risk for thrombosis and low bleeding risk. Ideal management of antiplatelet therapy when therapeutic anticoagulation is also required is challenging given the increased of bleeding and is the topic of ongoing large-scale clinical investigation.

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Physiological Roles of Leukocytes and Disorders

19

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Myeloid Cells

Myeloid cells are a subset of white blood cells or leukocytes. This subset includes neutrophils, eosinophils, monocytes, and basophils. These cells mediate host defense against infection and are part of the innate, as opposed to the adaptive, immune response. These cells contain granules ("granulocytes") and are derived from a common pluripotent myeloid stem cell precursor (Table 19.1).

Laboratory Studies

(a) An automated cell counter is used to determine the number of white blood cells (WBCs) in each microliter (μl) or cubic millimeter (mm^3) of the blood.
(b) These cells are further analyzed to determine the percentage of each subtype or the *relative proportion* of each cell type. The enumeration is known as the *differential cell count*.
(c) The *absolute number* of each circulating component of WBCs is determined by multiplying the percentage of each type of cell by the total number of white blood cells. When used to determine the absolute number of neutrophils, this value is called the "ANC" – absolute neutrophil count. Table 19.2 defines the terms that are used to describe variations in the WBC differential.

Table 19.1 Subsets of leukocytes

Myeloid-derived cells	Lymphoid-derived cells
Neutrophils	Natural killer cells
Basophils	T lymphocytes
Eosinophils	B lymphocytes
Monocytes	Plasma cells
Macrophages	

Subsets of Myeloid Cells

Neutrophils

Neutrophils are the most abundant leukocyte subset and account for approximately 70% of all circulating leukocytes in the blood. These cells have an essential role in host defense mechanisms through phagocytosis, release of granular contents and reactive oxygen species (ROS) and by trapping pathogens via web-like structures called nuclear extracellular traps (NETs). Given their role, neutrophils are needed continuously and have a high turnover rate.

Production

Neutrophils are generated in the bone marrow by a process called granulopoiesis. Hematopoietic stem cells in the bone marrow undergo a series of changes to give rise to the granulocyte-monocyte progenitor cells (GMPs). GMPs differentiate when stimulated by granulocyte colony-stimulating factor (G-CSF) or granulocyte-macrophage colony stimulating factor (GM-CSF). GMPs mature to neutrophils through a sequence of differentiation stages from myeloblasts, promyelocytes, myelocytes, metamyelocytes, and band cells to segmented neutrophils (Fig. 19.1). Each transition is heralded by acquisition of primary, secondary, and tertiary granules that contain specific bactericidal molecules. The transition from myeloblast to promyelocyte is marked by the appear-

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Table 19.2 Terms used to describe variations of the white blood cell differential

Term	Definition	Example
Absolute	Actual number of a specific type of white blood cells WBC/mm^3 or μl of the blood	ANC (number/μl) = total WBC count × % of neutrophils and bands in the differential; if the WBC count is 5000/μl and the percentage of neutrophils are 20%, ANC is 1000/μl
Relative	Relation of the number of a specific type of WBC to the total WBC count (the percentage shown in the WBC differential)	If the WBC count is 5000/μl and the differential shows that 90% of these cells are neutrophils, neutrophils are described as relatively increased (relative neutrophilia); "normal," or reference, neutrophil percentage is 50–80%
-philia, -cytosis	Indicate increased levels in the blood	Neutrophilia, eosinophilia, monocytosis, lymphocytosis
	-philia is used for granulocytes	
	-cytosis is used for lymphocytes and monocytes	
-penia	Suffix indicating decreased level in the blood; applicable to any WBC, to platelets, and to many non-hematologic substances that are normally present in the blood	Neutropenia, thrombocytopenia, insulinopenia

ANC absolute neutrophil count, *WBC* white blood cell

ance of primary granules that contain proteins such as neutrophil elastase (NE) and myeloperoxidase (MPO). These proteins exert potent bactericidal activity by breaking bacterial cell walls. Furthermore, these proteins aid in the formation of NETs by catalyzing the unraveling of DNA material [1, 2]. When myelocytes differentiate to metamyelocytes, they acquire secondary granules, which mostly consist of lactoferrin and lysozymes. Tertiary granules appear during band cell to segmented neutrophil differentiation. These granules contain gelatinase. The process from stem cell to postmitotic neutrophils takes approximately 4–6 days with an additional 5 days for mature neutrophils to be released in the circulation.

Bone Marrow Homing and Peripheral Functions

Once mature neutrophils form, they are tethered to the bone marrow via interactions between two chemokine receptors, CXCR4 and CXCR2, on neutrophils and their corresponding ligands (e.g., CXCL12) on the extracellular matrix [3, 4]. Other adhesion molecules such as integrin subunit alpha (ITGA4) and vascular cell adhesion molecule 1 (VCAM1) are also involved in tethering mature neutrophils. A decrease in CXCR4 expression leads to release of neutrophils from the bone marrow space. In addition to stimulating granulopoiesis, G-CSF also promotes the release of neutrophils from the bone marrow by decreasing the levels of expression of CXCR4 and CXCL12 [5].

Once in the bloodstream, neutrophils primarily exist in two forms. A small pool of neutrophils remains attached to the vessel wall (marginated neutrophils) whereas the majority of cells circulate in the blood. The migration of neutrophils into tissues is a multistep process that involves tethering and rolling along the endothelium, chemokine-induced neutrophil adhesion, and actin-dependent polarization and migration before final diapedesis (see below and Fig. 19.2) out of blood vessels [6, 7]. Endothelial cells near sites of inflammation become activated and express adhesion receptors, namely, P-selectin and E-selectin.

Neutrophils express their counterpart ligands which are glycosylated proteins P-selectin glycoprotein ligand-1 (PSGL-1) and leukocyte E-selectin ligand-1 [8]. The interaction between receptors and ligands helps capture neutrophils from the circulation, allows them to roll along the vessel wall, and eventually anchors them to the endothelial surface. Activation of neutrophils causes conformational changes of a group of cell surface receptors called integrins, which help neutrophils adhere firmly to the vessel wall. Integrins found on the neutrophil surface are heterodimeric complexes of two different molecules called the alpha- and beta-integrin subunits. Two integrins are of significant interest, lymphocyte function-associated antigen 1 (LFA1), also known as α1β2 (α1=CD11a, β2=CD18), and macrophage-1 antigen (Mac-1), also known as αMβ2 (αM=CD11b, β2=CD18), which upon activation bind onto endothelial cell surface molecules called intercellular adhesion molecule-1 (ICAM1) and ICAM-2. Binding of LFA1 and Mac-1 to ICAM molecules is key for firm adhesion of neutrophils to the vessel wall and primes these cells for the next step of crawling [9]. Once bound, neutrophils undergo a series of cytoskeletal changes that allow them to travel across the endothelium and crawl toward endothelial cell-cell junctions. In order to access sites of inflammation, neutrophils must traverse a layer of endothelial cells and the extracellular matrix either by migrating through individual cells (transcellular route) or between them (paracellular route). This process, termed diapedesis, is accomplished by numerous interactions between integrins and corresponding adhesion molecules and cytoskeletal changes [10–12] (Fig. 19.2). Once in tissues, neutrophils can kill pathogens through intracellular and extracellular mechanisms. Former include phagocytosis, which results in death of engulfed pathogens through NADPH-mediated reactive oxygen species (ROS). Extracellular mechanisms of pathogen elimination include release of antibacterial proteins (cathepsins, defensins, lactoferrin, and lysozyme) into the extracellular matrix and neutrophil extracellular trap (NET) formation.

NETs are web-like structures composed of DNA material (histones) decorated with antimicrobial neutrophil granules,

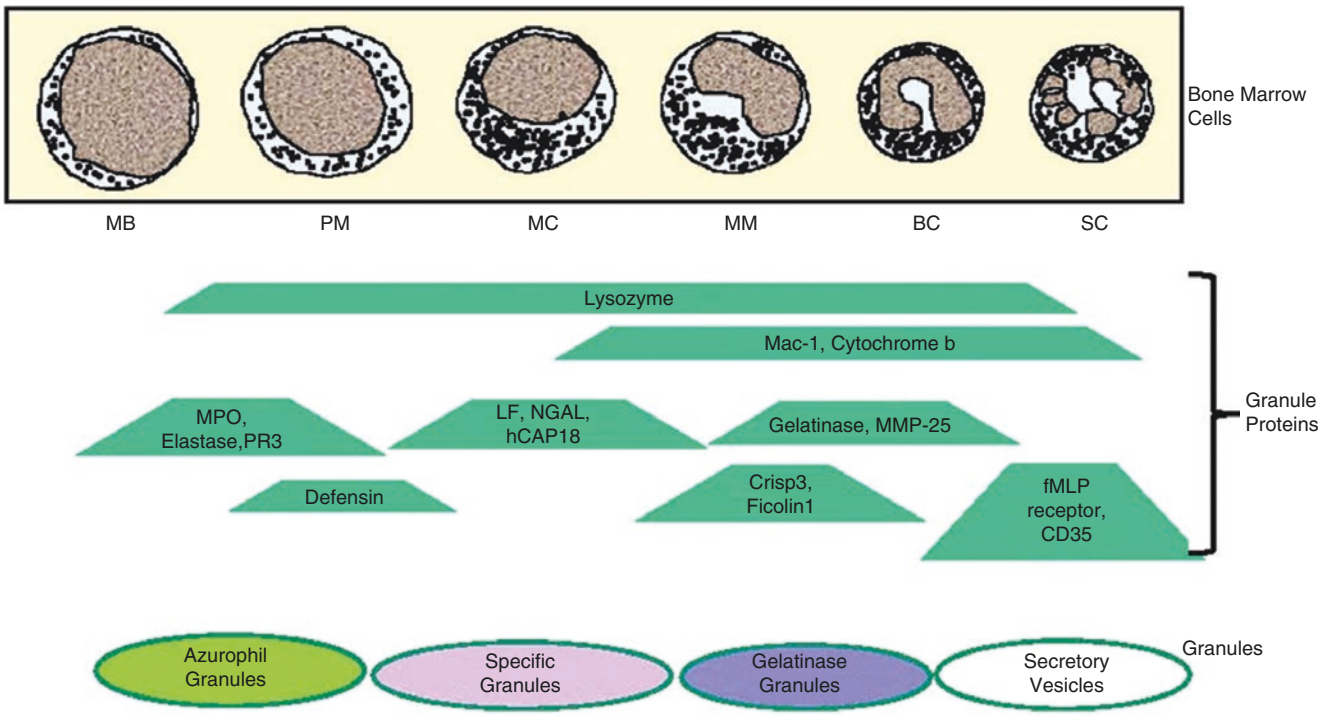

Fig. 19.1 Formation of -granules during maturation of neutrophils in the bone marrow. Transcription profiles for granule proteins are depicted during maturation of neutrophils from myeloblasts (MB) through promyelocytes (PM), myelocytes (MC), metamyelocytes (MM), band cells (BC), and segmented cells (SC). Four kinds of granules are seen in neutrophils: azurophilic, specific, gelatinous, and secretory vesicles. The contents of each of these granules are indicated on the figure

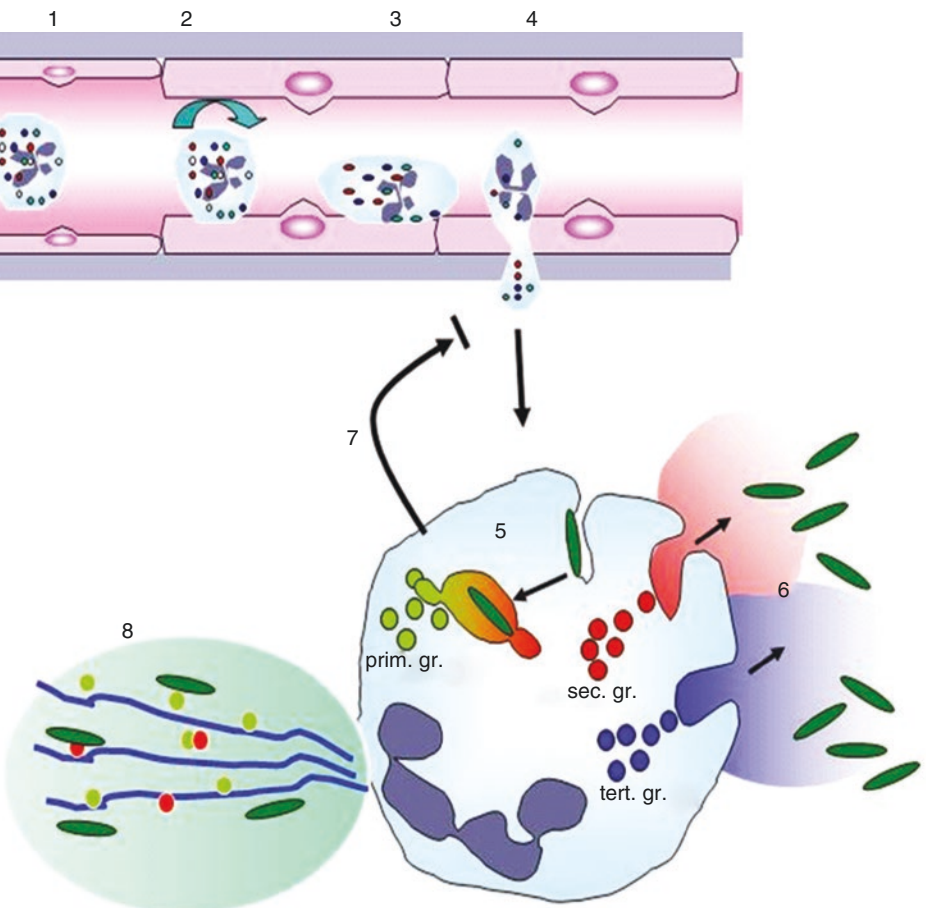

Fig. 19.2 Diapedesis of neutrophils into tissues. (1) Neutrophil passing through normal postcapillary venules. (2) Neutrophil rolling along activated endothelium during inflammation. (3) Neutrophil adhering firmly to activated endothelium. (4) Neutrophil migrating through the endothelial lining. (5) Uptake of microorganisms in a phagocytic vacuole. (6) Exocytosis of gelatinase granules and specific granules. (7) Negative feedback to dampen neutrophil diapedesis by secretion of resolvins. (8) Extrusion of DNA and formation of neutrophil extracellular traps (NETs) that bind and kill microorganisms

neutrophil elastase (NE), and myeloperoxidase (MPO) [1, 13]. A key enzyme in the formation of NETs is peptidylarginine deiminase 4 (PAD4). PAD4 is a nuclear enzyme that converts specific arginine residues to citrulline on histones [14]. Upon PAD4 activation, histones become citrullinated, which results in a change of net charge on histone (arginine is positively charged whereas citrulline is neutral). The change in overall charge leads to conformational changes resulting in unfolding of the DNA material, chromatin decondensation, and eventual extrusion from the nucleus [15]. The process of releasing NETs is termed NETosis and can result in cell death (suicidal NETosis) or preservation of neutrophils (vital NETosis) [16]. The stimulus seems to have an important part in determining which kind of NETosis is induced. Sterile inflammatory stimuli such as phorbol-12-myristate-13-acetate (PMA), autoantibodies, or cholesterol crystals induce suicidal NETosis which occurs after hours of stimulation [17]. Following activation of NADPH oxidase, ROS are produced and PAD4 is activated, resulting in chromatin decondensation. Subsequently, NE and MPO are translocated into the nucleus to promote further unfolding of chromatin, with resultant disruption of the nuclear membrane. Chromatin is then released into the cytosol, where it becomes decorated with granular and cytosolic proteins. Finally, NETs are released through disruption of the plasma membrane, and the neutrophil dies (Fig. 19.3) [18].

More recently, NETosis was shown to be induced independently of cell death. Whereas suicidal NETosis requires hours of stimulation and oxidant production, vital NETosis takes place within minutes of stimulation of neutrophils with bacteria or bacterial products, toll-like receptor (TLR) 4-activated platelets, or complement proteins [19]. PAD4 is activated without any need for oxidants [20] and induces chromatin decondensation. As in suicidal NETosis, NE is translocated into the nucleus to promote further unfolding of chromatin and nuclear membrane disruption. However, protein-decorated chromatin is expelled via vesicles, and the neutrophil stays alive for further functions (Fig. 19.4) [16]. Newer studies have also proposed that NETs can be produced with mitochondrial-derived DNA [21].

NETosis was initially described as an additional mechanism through which neutrophils help catch and kill bacteria. However, increasing evidence suggests that this process might also occur in noninfectious, sterile inflammation. In such settings, aberrant suicidal NETosis over time can be harmful to the host and lead to autoimmunity and venous thrombosis. This will be discussed in detail elsewhere in the chapter.

Elimination

The half-life of neutrophils in the bloodstream is anywhere between 13 and 19 h to 5.4 days [22]. This broad range likely reflects different techniques used to measure neutrophil kinetics. Senescent neutrophils are cleared from the circulation by Kupffer cells (resident macrophages) in the liver [23], by red pulp macrophages in the spleen [4], and by stromal macrophages in the bone marrow [24].

Disorders of Neutrophils

Disorders of neutrophils can be broadly classified into quantitative and qualitative defects. Both will be discussed in detail.

Quantitative Neutrophil Defects (Disorders that Affect Neutrophil Number)

Neutropenia in adults is defined as an absolute neutrophil count (ANC) of less than 1500/μL (in the newborn, less than 3000/μL). A neutrophil count of less than 500/μL is associated with increased risk of opportunistic fungal and bacterial infections. Conditions associated with neutropenia can broadly be classified into disorders related to decreased bone marrow production and disorders related to increased consumption and/or clearance.

Neutrophil Production Defects

Inherited disorders – hereditary disorders such as severe congenital neutropenia (SCN) and cyclic neutropenia (CN) – are often associated with a neutrophil count of less than 500/μL resulting in increased risk of infections. Both SCN and CN result from either inherited or acquired mutations in the neutrophil elastase or ELA2 gene [25]. These mutations affect NE protein production, which results in severe neutropenia due to maturation arrest of neutrophil precursors leading to their early apoptosis in the marrow [26]. Despite same gene mutations, neutropenia in SCN is persistent and leads to increased risk for leukemia transformation, whereas neutropenia in CN tends to occur in cycles of 21 days and does not carry the same leukemogenic risk. WHIM syndrome (WHIM) is an autosomal dominant inheritance immune deficiency that in most kindreds is caused by a gain in function mutation (hyperactivity with failure to downregulate) in CXC chemokine receptor 4 (CXCR4). The name of the syndrome is an acronym derived from major features of the disorder that include, but are not limited to, *W*arts, *H*ypogammaglobulinemia, recurrent bacterial *I*nfections, and *M*yelokathexis (apoptosis of mature myeloid cells in the marrow) [27]. Most, but not all, patients with WHIM are heterozygous carriers of mutations of CXCR4 that cause partial truncations of the carboxyterminal segment of this receptor. When the CXCR4 mutation connection to WHIM was first identified in 2003, it was considered to be the first example of a human disease mediated by dysfunction of a chemokine receptor. Treatment for all these disorders involves support of neutrophil counts with G-CSF. In contrast, constitutive or

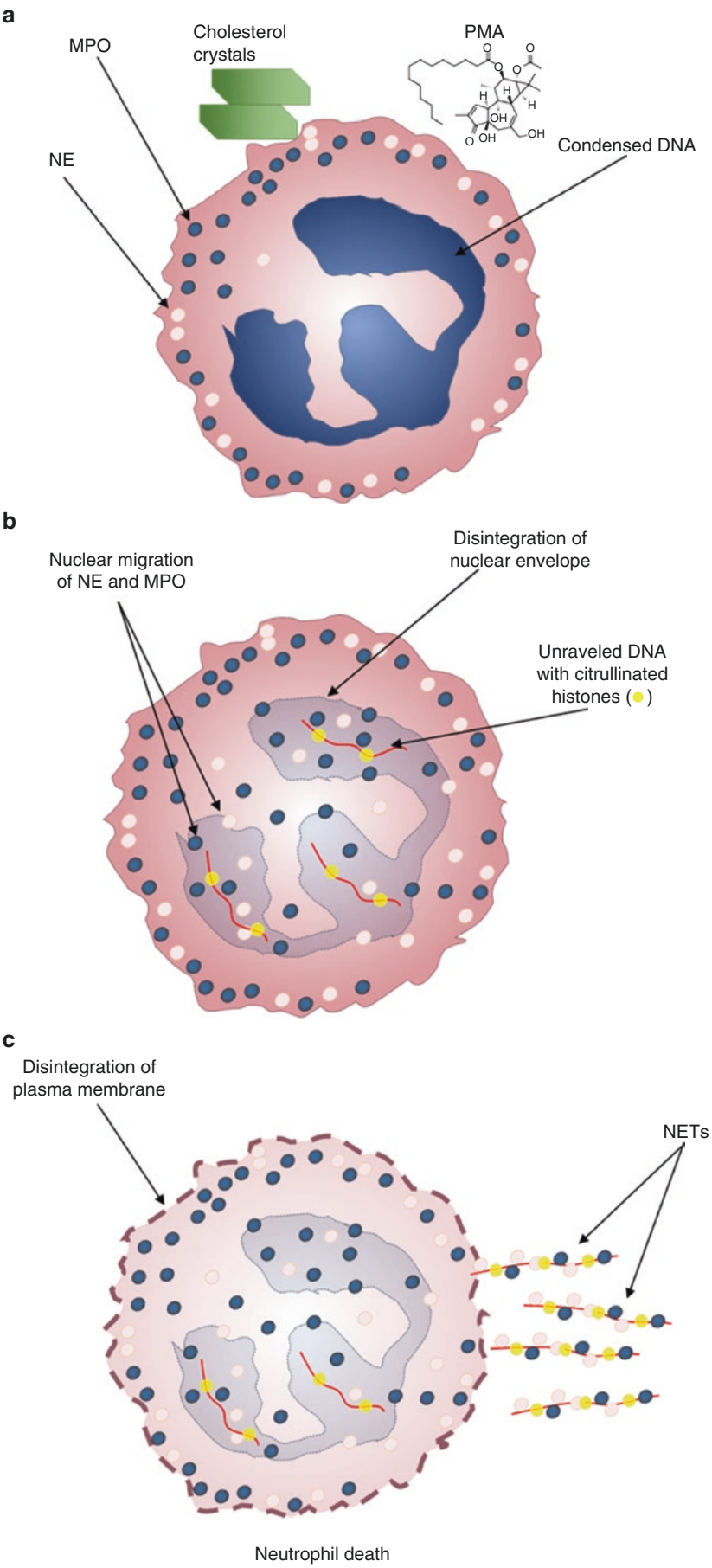

Fig. 19.3 Suicidal NETosis. (**a**) Sterile inflammatory stimuli such as PMA and cholesterol crystals induce ROS production and PAD4 activation, which results in chromatin decondensation. (**b**) Histones become citrullinated by PAD4, and neutrophil elastase (NE) and myeloperoxidase (MPO) migrate into the nucleus. (**c**) Citrullinated histones are subsequently expelled from the cell and become decorated with NE and MPO. NETosis leads to loss of the entire cell structure and cell death

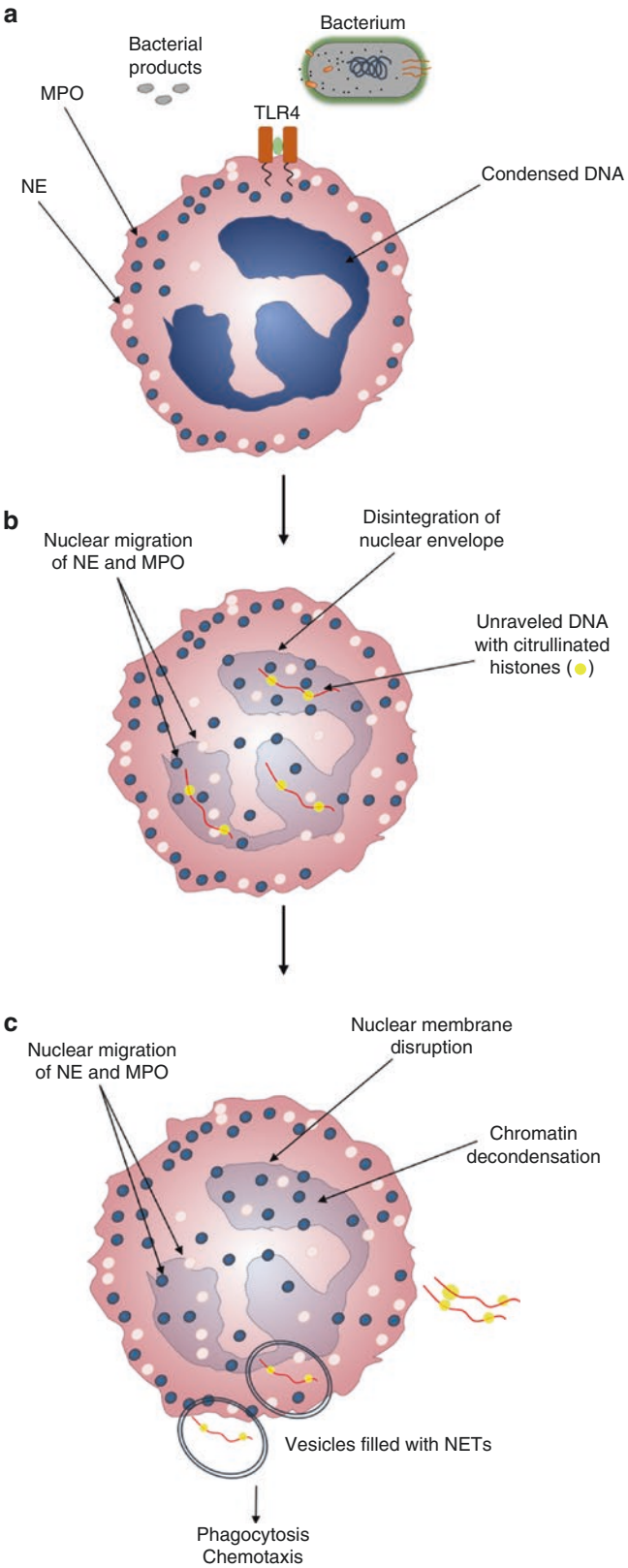

Fig. 19.4 Vital NETosis. (**a**) Pathogens are recognized by toll-like receptors and receptors for complement (C3). The nucleus loses its lobulated shape and becomes rounded. (**b**) The unraveled DNA is released into vesicles that bud from the nucleus. (**c**) These vesicles fuse with the cell membrane and release fragments of DNA coated with NE and MPO. The neutrophil stays alive for further functions, e.g., phagocytosis

Table 19.3 Medications that cause neutropenia

Antimalarial drugs (amodiaquine, chloroquine, quinine)
Antithyroid drugs (methimazole, propylthiouracil, thiocyanate)
Anticonvulsants (carbamazepine, felbamate, mesantoin, phenytoin, valproic acid)
Antibiotics (sulfonamides, ampicillin, ciprofloxacin, methicillin, cefotaxime)
Anti-inflammatory drugs (sulindac, sulfasalazine, dipyrone, ibuprofen, indomethacin, phenylbutazone, sulfapyridine)
Other drugs like chemotherapy agents, antihistamines like cimetidine and ranitidine, antipsychotics like clozapine, mianserin, chlorpromazine, allopurinol, ticlopidine, gold salts

ethnic neutropenia usually results in mild neutropenia and is not associated with increased risk of infections. This mild form of neutropenia is often seen in patients of Mediterranean and African ethnic background [28, 29]. The neutropenic phenotype in the African population results from polymorphisms of the Duffy antigen receptor complex (DARC) gene via unknown mechanisms [30]. These patients often do not require any treatment.

- Medications – drug-induced neutropenia is one of the most common causes of neutropenia. There are several medications that have been implicated in disrupting production of neutrophils. A list of common medications is listed in Table 19.3. These drugs can be broadly classified as those that cause a maturation arrest (such as methotrexate) or cause direct myeloid toxicity and myelosuppression (chemotherapy agents). Medications can cause isolated neutropenia (proton-pump inhibitors, clozapine) or pancytopenia (Bactrim and chemotherapy agents) [31]. It is unclear if any of these agents decrease the production of G-CSF or GM-CSF.
- Autoimmune diseases – autoimmune neutropenia is seen in diseases like rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), common variable immunodeficiency (CVID), and large granular lymphocyte leukemia (LGL). The mechanisms by which these diseases cause neutropenia vary. Potential mechanisms include decreased granulopoiesis by pro-inflammatory cytokines [32], antibodies against myeloid progenitor cells, antibody-mediated reduced marrow release, and increased apoptosis of mature neutrophils [33].
- Myelophthisis – about two thirds of the bone marrow space is devoted to the production of neutrophils. Mechanistically, diseases like lymphoproliferative disorders, myelodysplastic syndrome (MDS), leukemia, plasma cell dyscrasias, and cancer metastatic to the bone marrow occupy much of the marrow space and can therefore decrease neutrophil production. The displacement of normal hematopoietic precursors is termed myelophthisis. Furthermore, certain cancers (like myelofibrosis) may secrete cytokines that inhibit granulopoiesis and increase fibrosis in the marrow thus, causing cytopenias.

- Infections – viral illnesses including Epstein-Barr virus (EBV), human immunodeficiency (HIV), and hepatitis viruses lead to production of antibodies directed against neutrophils and their precursors and can lead to systemic neutropenia.

Disorders that Increase Neutrophil Destruction and Clearance

- Hypersplenism – splenic enlargement due to hematologic malignancies (myeloproliferative disorders, hairy cell leukemia, lymphoproliferative disorders, amyloidosis), portal hypertension (cirrhosis, Budd-Chiari syndrome), infections (Leishmania), or autoimmune diseases (Felty's syndrome) may lead to sequestration and premature neutrophil clearance from the circulation.
- Drugs – drugs and their intermediates may bind to the surface of neutrophils and act as haptens leading to the formation of antineutrophil antibodies and neutrophil destruction by opsonization [31].
- Sepsis – endotoxins released during overwhelming bacterial infections cause a consumptive effect on neutrophils.
- Artificial surfaces – neutrophils are often removed via hemodialysis as cells adhere to artificial surfaces [34].

Neutrophilia

Neutrophilia is defined as a neutrophil count that exceeds the upper limit of normal. It can be caused by either primary or secondary causes.

Primary/Clonal Neutrophilia

Primary neutrophilia refers to increased neutrophil count due to abnormal neutrophil production. Myeloproliferative disorders including chronic myelogenous leukemia (CML) (see Chapter 25), chronic neutrophilic leukemia, myelofibrosis, Down syndrome, and chronic idiopathic neutrophilia are associated with increased neutrophil production.

Secondary Neutrophilia

Neutrophilia due to ongoing processes such as smoking, infection, inflammation, medications, stress, and malignancy is called secondary or reactive neutrophilia. It is estimated that heavy smoking (two packs per day) can cause a 27% increase in total leukocyte count with almost doubling in absolute neutrophil count [35, 36].

Infection can lead to increased release of band forms and mature neutrophils from the bone marrow and demargination of neutrophils attached to the vessel wall [37].

Chronic inflammatory conditions like Crohn's disease stimulate granulopoiesis and lead to leukocytosis [38].

Medications like corticosteroids and G-CSF can also cause neutrophilia. Steroids primarily cause demargination of neutrophils that are adhered to the vessel wall, but they can also increase cell release from the bone marrow by stimulating production of G-CSF.

Anxiety, exercise, hypoxia, and stress can also cause mild neutrophilia by means of demargination under the influence of epinephrine [39].

Qualitative Neutrophil Defects (Disorders that Affect Neutrophil Function)

Neutrophils have a central role in clearing infections. They achieve this function through carefully orchestrated events that allow them to reach sites of infection from the bloodstream. Disorders of neutrophil function occur when key steps such as recruitment, adhesion, migration, and/or degranulation are impaired due to either inherited or acquired defects.

Chediak-Higashi syndrome (CHS) is a rare autosomal recessive disorder characterized by oculocutaneous albinism along with recurrent pyogenic infections. It is thought to result from a mutation in CHS1 gene that is responsible for regulating lysosome-related organelle size and secretion [40]. The absence of this gene results in large primary granules, which are ineffective in intracellular pathogen killing and neutrophil migration [41].

Leukocyte adhesion deficiency is a group of disorders that primarily result from lack of expression of beta-integrins or CD18 which facilitate neutrophil binding to the surface of endothelial cells. These disorders lead to recurrent bacterial and fungal infections due to lack of neutrophil recruitment and migration [42].

Chronic granulomatous disease (CGD) is a rare disease characterized by recurrent bacterial and fungal infections. Five mutations (CYBA, CYBB, NCF1, NCF2, and NCF4) have been implicated in the pathogenesis of CGD [43]. The proteins produced from these genes are part of an enzyme complex called nicotinamide adenine dinucleotide phosphate oxidase (NADPH), which participates in production of superoxide. Superoxide and its downstream products, hydrogen peroxide and hypochlorous acid, are crucial for bacterial killing. Mutations in any of the aforementioned genes lead to defective NADPH oxidase activity and impaired phagocytosis of microorganisms [44]. Table 19.4 provides a summary of disorders causing quantitative and qualitative neutrophil defects.

In addition to specific genetic mutations, acquired condition such as aberrant NETosis is thought to play a major role in diseases including thrombosis, autoimmune conditions like systemic lupus erythematosus (SLE), and cancer. NETs contribute to thrombosis through several mechanisms. First, proteins decorating NETs, namely, neutrophil elastase, inhibit tissue factor pathway inhibitor (TFPI) and thus enable unopposed coagulation [45]. Second, histones can directly stimulate platelet aggregation [46]. Finally, NETs also provide a scaffold for platelets and RBCs and localize proteins like tissue factor, von Willebrand factor (vWF), and Factor

Table 19.4 Structural and functional defects in neutrophils

Step	Disease	Molecular defect
Rolling	Leukocyte adhesion deficiency (LAD-2)	Sialyl Lewis^x carbohydrate abnormalities with fucosylation defect due to defective protein necessary for transport of GDP-fucose in Golgi. Generalized loss of expression of fucosylated glycans on cell surfaces
Tight adhesion and phagocytosis	LAD-1	Loss or diminished β_2-integrin (CD18) expression
Defective neutrophil and platelet activation	LAD-3	Defection activation pathway of β2 integrin due to mutations in kindlin-3, an integrin-binding protein
Migration and degranulation	Specific granule deficiency	Deficiency of the myeloid-specific transcription factor C/EBPε
Degranulation	Chediak-Higashi syndrome	Lysosomal trafficking regulator protein CHS1 (LYST) gene leading to a defect in granule morphogenesis
Oxidative burst	Chronic granulomatous disease	NADPH oxidase activation defect due to deficiencies in gp91^{phox} (65%), p22^{phox} (5%), p67^{phox} (5%), p47^{phox} (25%), or p40^{phox} (rare)
	Myeloperoxidase deficiency	Loss of HOC l production; defective posttranslational processing of MPO

NADPH reduced nicotinamide adenine dinucleotide phosphate

XII leading to fibrin formation and amplification of the developing thrombus [47, 48].

Recent studies show that neutrophils and NETs play an important role in both the activation and resolution of inflammation. Uptake of senescent neutrophils by dendritic cells (DCs) enhances T-cell recruitment [49]. Also, neutrophils produce cytokines that are essential for B-cell development [50]. It has been hypothesized that when NETs are formed, they expose the immune system to autoantigens. Therefore, aberrant NET formation and its poor clearance over time create enough exposure to form autoantibodies [51]. Indeed, SLE where antibodies are directed against double-stranded DNA [52], or small-vessel vasculitis where antibodies are produced against myeloperoxidase (MPO), is thought to arise as the result of dysfunctional NETosis [53]. Aberrant NETosis has also been implicated in improper wound healing in patients with diabetes [54]. In contrast to the above, NETs also help resolve inflammation by degrading chemotactic cytokines [55].

Lastly, neutrophils exert a dual role in tumorigenesis. Unregulated production of reactive oxygen species (ROS) and proteases by neutrophils induces epithelial damage, thereby contributing to tumor-promoting inflammation [56]. Neutrophils promote cancer progression by reprogramming senescent cancer cells into proliferating cells by direct transfer of NE, which activates intracellular cell signaling pathways important in cell proliferation. In addition, neutrophils can assist cancer cells in escaping cytotoxic T cells by producing chemokines that deter T-cell growth [57]. Lastly, neutrophils participate in remodeling of the extracellular matrix and induce angiogenesis that helps in tumor metastasis [58]. In some context, neutrophils can also limit tumor growth. Hypoxia in the tumor environment induces expression of ligands that attract antitumor neutrophils [59]. It is unclear if the different actions are mediated by different subsets of neutrophils or whether these cells are very adaptive and exhibit variable responses depending on the local microenvironment.

Eosinophils

Eosinophils comprise a very small proportion of circulating leukocytes. Unlike neutrophils, much about their development and function still remains unknown. Eosinophils also arise from hematopoietic stem cells in the marrow. Pluripotent cells under the influence of multiple transcriptional factors undergo a series of programmed events to commit to the myeloid lineage. Expression of CEBP (CCAAT/enhancer-binding protein family) proteins such as CCAAT/enhancer-binding protein alpha (C/EBPα) and CCAAT/enhancer-binding protein epsilon (C/EBPε) in addition to GATA-1 and PU.1 are important for eosinophil lineage commitment [60–63]. Further differentiation occurs under the stimulation of GM-CSF and two cytokines, namely, IL-3 and IL-5 [64]. Expression of IL-5 is also responsible for the release of eosinophils from the marrow into the peripheral circulation [65]. Eosinophils exist in the circulation for about 18 h before leaving to reside in tissues including mammary glands, gastrointestinal tract, uterus, lymph nodes, and thymus [66]. Interestingly, eosinophils transmigrate the endothelium via the same processes as neutrophils through rolling, adhesion, and diapedesis [67].

Eosinophils contain four different types of granules, namely, eosinophil cationic protein (ECP), myeloid basic protein (MBP), eosinophil-derived neurotoxin (EDN), and eosinophil peroxidase (EPO). These granules participate in defenses against various pathogens like parasites, bacteria, fungi, and viruses and are capable of inducing tissue damage and dysfunction [68]. Interestingly, eosinophils are also capable of forming NETs [69]. Eosinophils are normally thought to be host defense against parasitic infections. However, there is a growing body of evidence that their roles are quite complex and varied. Eosinophils are now considered to participate as antigen-presenting cells promoting T-cell proliferation [70]. They can also migrate to the lymph nodes to recruit more T cells via a process called trafficking [71, 72]. In addition to interacting with T cells, eosinophils

also prime B cells for production of IgM against pathogens and interact with DCs, mast cells, and neutrophils [67]. In addition, they are also now thought to play a role in maintaining uterine environment during pregnancy [73].

Diseases that Affect Eosinophil Number

Eosinopenia is a relatively benign finding with eosinophil count less than 400 cell/μL. The levels could decrease secondary to steroid use or infection.

Eosinophilia is characterized by an absolute eosinophil count of greater than 500 cells/μl. Eosinophilia can be further divided clinically as blood or tissue eosinophilia, which can exist either alone or together. Common diseases associated with blood eosinophilia include parasitic infections and allergic diseases such as drug or food allergies, asthma, adrenal insufficiency, HIV, and bone marrow disorders like chronic eosinophilic leukemia [67, 74]. Diseases associated with tissue eosinophilia include atrophic gastritis, eosinophilic esophagitis, T-cell lymphoma, and eosinophilic pneumonia [75] [67, 74]. A group of disorders are associated with blood hypereosinophilia (defined as a peripheral blood count greater than 1500 cells/μl) and tissue infiltration leading to organ dysfunction. These disorders are collectively termed hypereosinophilic syndrome (HES) [76]. Cardinal features include eosinophilia on more than two occasions over a 6-month period and organ dysfunction due to eosinophil infiltration. These disorders can sometimes be associated an interstitial chromosomal deletion of the CHIC2 domain on chromosome 4q12 generating an activated tyrosine kinase fusion protein, FIPIL1-PDGFRA [76]. The presence of this mutation is also indicative of response to tyrosine kinase inhibitors like imatinib [77].

Disorders of Abnormal Eosinophil Function

Abnormal trafficking of eosinophils in tissues has been implicated in diseases like asthma, atopic dermatitis, and esophageal esophagitis [74]. In asthma, abnormal degranulation releases myeloid basic protein (MBP), which in turn increases smooth muscle reactivity. MBP can also trigger degranulation of mast cells and basophils, also involved in disease pathogenesis [78]. Additionally, eosinophils generate large amounts of leukotrienes that lead to increased vascular permeability, mucus secretion, and smooth muscle constriction [74].

Basophils and Mast Cells

Basophils and mast cells are the circulating and tissue-bound forms of cells, respectively, which are related but develop from separate precursor cells. Basophils are easily identified by their dark, dense granularity on Wright-Giemsa stain. These cells are present in the blood in very small numbers. Mast cells arise from CD34+/c-Kit+/CD13+ pluripotent hematopoietic cells in bone marrow and have a different origin than basophils. They share cytoplasmic basophilic granules, high-affinity IgE receptors, and histamine release upon stimulation like basophils but are considered distinct. Mast cells reside in connective tissue. Both types of cells are believed to play a critical role in host defense against parasites and participate in atopic processes. Basophils increase in myeloproliferative disorders.

Disorders of basophils

- A low basophil count is associated with glucocorticoid treatment and hypersensitivity reactions.
- The basophil count is increased in patients who have allergic conditions, infection, endocrinopathy, and myeloproliferative disorders (e.g., cell-mediated lymphotoxicity, polycythemia rubra vera, myeloid metaplasia, essential thrombocythemia).

Disorders of Mast Cells

- Systemic Mastocytosis is a disorder associated with mast cell infiltration of the skin or other organs and is associated with symptoms related to excess histamine. Clinical signs include urticaria, hives, and dizziness. Systemic mastocytosis is due to a gain-of-function mutation in *c-kit*, kit D816V. Two other mutations, *FIP1L1-PDGFRA* (platelet-derived growth factor receptor A) and kit F522C, have been associated with this disorder as well. *FIP1L1-PDGFRA* has tyrosine kinase activity and is also associated with eosinophilia, as described above.

Monocytes

Once monocytes reach the tissue, they differentiate into macrophages and dendritic cells (DCs) and can remove microorganisms and noxious agents. Like neutrophils, these cells phagocytize pathogens. They also generate toxic oxygen metabolites that have a role in killing microorganisms. Monocytes are essential in antigen presentation to T lymphocytes. They also are necessary for tissue repair and remodeling. An increase in monocytes (monocytosis) occurs in tuberculosis and neoplasms.

Disorders of monocytes parallel those seen with neutrophils.

(A) The average circulating monocyte count is 300/μl and can range from 0 to 800 cells/μl.
(B) *Monocytopenia* occurs in response to stress, endotoxemia, and after glucocorticoid administration.
(C) *Monocytosis* is present when the absolute monocyte count exceeds 800/μl. It occurs in chronic inflammatory states such as in:
   1. Myelodysplastic syndromes
   2. Neutropenic states (e.g., cyclic neutropenia)
   3. During the recovery phase of agranulocytosis
   4. Exacerbations of lymphoma
   5. Patients who have undergone splenectomy
   6. Subtypes of leukemia

7. Response to infection (e.g., acute bacterial, tuberculosis, listeria, cytomegalovirus, tuberculosis, subacute bacterial endocarditis, syphilis, fever of unknown origin)
8. Patients who have underlying inflammatory disease, such as systemic lupus erythematosus, rheumatoid arthritis, temporal arteritis, polyarteritis, gout, ulcerative colitis, regional enteritis, and sarcoidosis

Lymphocytes

Lymphocytes are derived from lymphoid lineage, which consist of natural killer (NK) cells, T lymphocytes, B lymphocytes, and plasma cells, all of which participate as the foot soldiers of the immune system. Lymphocytes can be divided into innate and adaptive immune cells. The innate cells include natural killer cells, and the rest comprise of adaptive cells, each of which will be discussed below.

Natural Killer Cells

Natural killer (NK) cells are best known for host defense against tumor and virus-infected cells. However, there is growing appreciation as to their involvement in other vital functions including cytokine and chemokine production, regulation of immune cell homeostasis, and preservation of pregnancy [79, 80].

Ontogeny of NK Cells

NK cells develop from hematopoietic stem cells in the bone marrow, although NK progenitor cells are also present in peripheral tissues such as the uterus, liver, and spleen. Human NK cells develop from CD34+ hematopoietic stem cells. CD34 and CD7 expression as well as cytokines including stem cell factor, IL-2, and IL-7 are essential for completion of the differentiation process [81]. NK cells differentiate through a continuum of progenitor cells to eventually give rise to CD56-bright and CD56-dim NK cells (the most mature cell), which retain different abilities to secrete cytokines and kill target cells [82]. CD56-bright cells are more potent chemokine and cytokine producers and are mostly present in lymphoid tissues. CD56-dim NK cells have more cytotoxic activity and comprise about 95% of the circulating NK cells in the blood [83].

NK Cell Functions

NK cells kill target cells primarily through three different mechanisms. One pathway involves proteins released from granules such as perforin and granzymes that kill cells by perforating target cell membrane thus inducing cell lysis or apoptosis [84]. A second mechanism is through secretion of cytokines that aid other immune cells. Third, NK cells are known to generate interferon-γ (IFN-γ), TNF (tumor necrosis factor), GM-CSF (granulocyte-macrophage colony-stimulating factor), and MIP-1α (macrophage inflammatory protein-1α) [85]. IFN-γ is helpful in T-cell differentiation [86], and MIP-1α aids macrophages by enhancing their antimicrobial function. Other pathways involve engagement of TNF family receptors on target cells, with their corresponding ligands (such as Fas ligand, TRAIL, and TNF) expressed by NK cells [87], leading to cell death.

Engagement of NK cells on their targets is mediated by NK cell receptors. These receptors recognize a quantitative change in major histocompatibility complex (MHC) molecules induced as a result of infection [88]. There are at least three known types of receptors on NK cells that recognize MHC molecules. One family belongs to the immunoglobulin (Ig) superfamily and is composed of killer cell immunoglobulin-like receptors (KIR) [89]. The second family is structurally Ig-like, named Ig-like transcripts (ILTs). They are also called LIRs for leukocyte Ig-like receptors. The third family consists of C-type lectin receptors [90]. The major inhibitory receptors are KIR family receptors and C-type lectin-like Ly49 receptors. Activating NK receptors are NKp30, NKp44, NKp46, and the C-type lectin-like receptor (CTLR) NKG2D [91]. These receptors act in concert and can exert either activating or inhibitory influences on the cytotoxic activity of NK cells.

NK cells also have a role in tumor surveillance. In patients who underwent allogeneic hematopoietic progenitor cell transplantation, the presence of donor NK cells induced a potent graft versus leukemia effect due to lack of HLA class I ligands on myeloma cells of the recipient to donor inhibitory KIR receptors [92]. This graft versus leukemia effect led to an overall increase in activation and cytotoxic effect on tumor cells by donor NK cells. Finally, NK cells also create an immune-tolerant state in the uterus during pregnancy and assist in remodeling of placental vasculature [93].

Disorders Mediated by Abnormal NK Cell Number

Classical NK cell deficiency (CKND) is defined as the absence of NK cells in the blood and increased susceptibility to viral infections [94]. There are at least two known subtypes of this disease, CNKD1 and CKND 2. CNKD1 is caused by haploinsufficiency of GATA 2, a hematopoietic transcription factor that promotes survival and maintenance of hematopoietic cell subsets [95]. CKND2 stems from a partial deficiency in MCM4 gene expression that encodes the mini chromosome maintenance (MCM) complex member 4. MCM4 enables helicase function during DNA replication and is important for all dividing cells. Patients with this disease often have a syndrome of NK cell deficiency, adrenal insufficiency, and intrauterine growth retardation [96].

NK cell leukemia results from clonal expansion of mature NK cells. This type of leukemia is very aggressive and associated with an extremely poor survival [97, 98].

Chronic NK lymphocytosis (CNKL) is an indolent NK cell disorder associated with increased NK cell number. Manifestations include skin lesions, vasculitis, and neuropathy [99]. It has been postulated that the lack of inhibitory KIR expression leads to unopposed NK-cell activation and consequent vascular inflammation [100].

Disorders Mediated by Abnormal NK Cell Function

Clinical studies show that low NK cell activity may lead to increased risk of tumors [101].

Hemophagocytic lymphohistiocytosis (HLH), also referred to as familial erythrophagocytic lymphohistiocytosis, is a disorder of activated histiocytes and T cells, anemia, thrombocytopenia, and fever. It results from uncontrolled activation of T cells producing IL-2, tumor necrosis factor-alpha, and interferon-gamma and has been associated with impaired or defective NK cell function [102]. Finally, abnormal trafficking of NK cells to tissues has also been implicated in the pathogenesis of autoimmune diseases like psoriasis, rheumatoid arthritis (RA), multiple sclerosis (MS), and lupus (SLE) [103].

B-Cell Lymphocytes

B lymphocytes along with T lymphocytes are involved in adaptive immunity.

Ontogeny of B Cell

B cells begin their development in the bone marrow. B cells originate from the common lymphocyte progenitor (CLP) cell. They differentiate through five defined stages of B-cell maturation before egressing from the bone marrow: from CLP to pro-B cell (early and late) to pre-B cell (large) to pre-B cell (small) to immature B cell. Immature B cells leave the bone marrow to complete their developmental stages into mature B cells in the lymph nodes and spleen. Common lymphocyte progenitor cells are irreversibly committed to B- or T-cell differentiation; however, more terminal stages of B-cell differentiation require specific bone marrow niches and availability of certain growth and differentiation factors. B-cell precursors downstream of CLP become dependent on stromal cell-derived factor-1 (SDF1) chemokine and its receptor CXCR4 [104]. The SDF-1 and its receptor are responsible for specific B-cell precursor biologic activities. Other important molecules for the function of B cells are the early B-cell factor (EBF), the FLT3 ligand, and the chemokine ligand CXCL12 [105]. These chemokines induce terminal deoxynucleotidyl transferase (TdT) and recombination-activating genes 1 and 2 (RAG-1 and RAG-2) genes that are essential for production of antibodies and B-cell receptors (BCR) [105]. Under the direction of interleukin-7 (IL7) produced by the bone marrow stromal cells, pro-B cells mature [106]. The early pro-B cell does not express immunoglobulins on its surface but starts to produce the earliest sections of immunoglobulin heavy chains. The completion of heavy chain formation hallmarks the late pro-B-cell stage. An important enzyme in this stage is Bruton's tyrosine kinase (BTK), which aids in the maturation to pre-B-cell stage [107].

Pro-B cells become pre-B cells when they express the M heavy chain immunoglobulin (precursor of immunoglobulin M) along with surrogate light chains (V-pre B and λ-like) and two other proteins called Ig-α and Ig-β [108]. This set is called the pre-B receptor. Surrogate light chains are similar to light chains on an antibody except that they are shared among all pre-B cells. The early immunoglobulin receptor is too short to transduce a signal into the cytoplasm. The surrogate light chain helps transmit such signals via ITAMs (immunoreceptor tyrosine activation motifs), which become phosphorylated in response to antigen binding [109]. The phosphorylation step arrests cells from creating recombinant heavy chains, a process called allelic exclusion, and drives cloning of pre-B cells with the same M-chain. Since dividing cells are large, this stage is called the large pre-B cell. The small pre-B-cell stage is heralded by formation of recombinant light chains. Once the light chains combine with the M-chain of surface immunoglobulin (this forms surface IGM or B-cell receptor (BCR)), the cells are termed immature B cells. These cells bind to self-antigens in the bone marrow and are eliminated by a process called negative selection. Immature B cells eventually mature and express immunoglobulin D along with other functionally important receptors [110].

Immature B cells discharged from the bone marrow relocate to the spleen where they undergo further maturation. B-cell egress from bone marrow is thought to be regulated by sphingosine-1-phosphate (S1P) [111] and cannabinoid receptor 2 (CNR2) [112]. Once in the spleen, B cells differentiate into either marginal zone (MZ) or follicular (FO) cells. The differentiation of B cells into either cell is dictated by expression of certain transcription factors and interaction with nearby cells. MZ cells develop from B cells after induction of neurogenic locus notch homolog protein 2 (NOTCH2). The expression of this protein allows for interaction with the vascular tissue within the spleen and leads to further differentiation [113]. FO B cells arise through a pathway dependent on Bruton's tyrosine kinase (BTK) induced by B-cell receptor-mediated signals [114]. FO cells are capable of creating long-lasting antibodies against antigens. MZ B cells are retained in the spleen, while FO B cells exit the spleen to populate other lymph nodes and to reside in organs like the lungs [114].

Classical humoral immunity results in high-affinity antibody production that is produced by a process called somatic

hypermutation and isotype switching. This allows B cells to produce antibodies that are specific to antigens and also creates long-lasting memory cells. This requires three main components: (1) an antigen, (2) corresponding receptors, and (3) cytokine and costimulatory signals [115]. MZ cells express toll-like-receptors that, when bound to their antigen and along with cytokines, rapidly differentiate into plasma cells producing non-specific antibodies. Follicular cells as mentioned before are involved in more specific antibody production. Once an antigen is bound to the B-cell receptor, it transduces signals to jumpstart essential gene transcription. In addition, antigens that are internalized by B cells are broken down to smaller peptides that eventually become displayed on the cell surface through MHC class II. CD4-positive T cells recognize these digested peptides and provide stimulatory signals to help B cells proliferate and differentiate [116]. It is also worth mentioning that only a few subsets of B cells were discussed in this chapter. We acknowledge that although there are several other subsets of B cells including memory cells and plasma cells, a detailed review of these cells is beyond the scope of this chapter.

Disorders of B-Cell Number and Function

Hereditary causes of low B cells include conditions such as Bruton's agammaglobulinemia and selective IgA deficiency. X-linked severe combined immunodeficiency (SCID) will be described in the T-cell section.

Bruton's agammaglobulinemia or X-linked agammaglobulinemia is the result of a mutation of Bruton's tyrosine kinase (BTK), an enzyme critical for the maturation of early B cells. The resultant defect leads to severe deficiency of antibody-generating B cells [117].

Selective IgA deficiency results from mutations in TACI (transmembrane activator and calcium modulator and cyclophilin ligand interactor), which leads to B cells that are able to express IgM and IgD but are unable to switch their isotype to IgA. It is the most common immunodeficiency disease. The inheritance of this disorder is variable and has often been associated with coinheritance of other immunodeficiency states [118].

Diseases that cause an increase in B-cell populations most often result from B-cell neoplasms and are described later in this chapter.

T Cells

T lymphocytes are the main cytotoxic effectors cells of the immune system.

Ontogeny of T Cell

T lymphocytes are also the progeny of common lymphocyte progenitor cells (CLP). Committed lymphoid progenitors egress the bone marrow and migrate to the thymus. The outer periphery of the thymus is called the cortex whereas the innermost section is called the medulla [119]. Early committed T cells lack the expression of T-cell receptor (TCR), a protein composed of an alpha and beta chains and are called double-negative (DN) thymocytes. Akin to B-cell maturation, DN thymocytes mature through four well-orchestrated stages of differentiation. The alpha beta-TCR+CD4+CD8+ double-positive (DP) thymocytes then interact with cortical epithelial cells that express a high density of MHC class I and class II molecules associated with self-peptides. The fate of the DP thymocytes is decided by the intensity of signaling that occurs upon binding of TCR to these self-peptide–MHC complexes in the cortex and medulla. Too little signaling through TCR in the thymocytes in the cortex results in delayed apoptosis (death by neglect). Too much signaling through TCR in the thymocytes in the medulla can promote acute apoptosis (negative selection). The appropriate level of TCR signaling initiates effective maturation (positive selection). Thymocytes that express TCRs and which recognize self-peptide-MHC-class I become CD8+ T cells, whereas those that express TCRs that bind self-peptide-MHC-class II ligands become CD4+ T cells. CD8+ cells are one of the main cytotoxic cells, whereas CD4+ cells are termed helper cells. Once these cells complete their education, they are exported from the medulla to peripheral lymphoid sites [120].

T cells constantly recirculate between secondary lymphoid organs and the blood to increase the likelihood of encountering an antigen-presenting cell (APC). T cells express adhesive molecules that help slow it down to allow for TCR binding to MHC molecules. Once a T cell recognizes a peptide attached to a MHC molecule, another set of surface protein interaction (B7 on APC and CD28 on T cell) provides the costimulatory signaling that activates T cells. T-cell activation leads to expression of more ligands that can increase the costimulatory signaling but also of inhibitory ligands such as CTLA4. The balance of these activating and inhibiting ligand-receptor interactions creates immune homeostasis [121].

Activated T cells synthesize IL-2 that leads to clonal expansion. The activation leads to three classes of effector cells that are specialized to deal with three different kinds of pathogens. CD8+ T cells kill infected cells that display pathogen peptides on class I MHC. CD4+ T helper 1 (Th1) cells activate macrophages, which in turn showcase peptides displayed on class II MHC. They also activate B cells to produce opsonizing antibodies. CD4+ T helper 2 (Th2) cells activate B cells that have used B-cell receptors to internalize specific antigen and display peptides on class II MHC [122].

Cytotoxic T cells are specialized in killing cells that harbor cytosolic pathogens such as parasites and viruses. The MHC-TCR binding allows the T cell to release perforins and

granzymes that help perforate the cell membrane and induce apoptosis of the target cell [123, 124]. Th1 CD4+ T cells also produce IL-2 and TNFb, which activate macrophages to stimulate cellular immunity and inflammation. Th1 cells also secrete IL-3 and GM-CSF to stimulate bone marrow to produce more leukocytes and signal B cells to produce opsonizing antibodies [125]. Helper or Th2 CD4 T cells activate naïve B cells to divide and secrete IgM; Th2 cells also secrete IL-4, IL-5, and IL-6, which stimulate opsonizing antibody production by B cells [125].

It is worth mentioning that not all the cells are effectors cells. A subset of CD4-positive cells known as the T-regulatory cells (Tregs) are crucial for maintaining self-tolerance and therefore preventing autoimmune diseases [126]. These cells are characterized by the expression of the transcription factor, Foxp3.

Disorders of Abnormal T-Cell Quantity

Hereditary immunodeficiency syndromes like X-linked severe combined immunodeficiency (SCID) and Omenn syndrome result in reduced number of T cells. SCID can present in several forms. It can be X-linked or due to adenosine deaminase deficiency (ADA). X-linked SCID is related to mutations in IL2RG, which encodes for gamma chain, the basis of most cytokines such as IL-2 and IL-7. The mutated IL-2 and IL-7 are unable to participate in T-cell development resulting in T-cell lymphopenia and in the absence of proper T-cell stimulation [127]. ADA deficiency results in defective nucleotide production that is essential for lymphocyte proliferation. These events result in combined T- and B-cell lymphopenia [127].

Omenn syndrome is a form of severe combined immune deficiency, which results from defective RAG (recombination-activating gene). This leads to ineffective lymphopoiesis resulting in severe reduction in the number of B and T cells. However, unlike X-linked SCID, a small population of self-reactive Th2 helper cells survive and proliferate, resulting in unchecked production of cytokines. The uncontrolled production of cytokines leads to the manifestations of desquamating skin disease, alopecia, and other autoimmune phenomena associated with this disease [128].

Drugs such as chemotherapy agents can also cause acquired lymphopenia by affecting the population of precursor and mature T cells.

Perhaps the most well-known disease that causes acquired deficiency in T cells is *acquired immunodeficiency syndrome* (AIDS), which is caused by the human immunodeficiency virus (HIV). HIV infects the CD4+ T helper cells and reduces their number by either inducing apoptosis or by CD8-positive cells attacking infected cells [129]. Diseases that cause an increase in T-cell number are often due to T-cell neoplasms such as T-cell lymphoma. These will be reviewed later in the chapter.

Disorders that Affect T-Cell Functions

IPEX (*i*mmune dysregulation, *p*olyendocrinopathy, *e*nteropathy, *X*-linked) is a rare, often fatal, X-linked immune dysregulatory disorder that typically presents during infancy with a triad of enteropathy, autoimmune endocrinopathy, and dermatitis [130]. IPEX is caused by mutations in the gene for the transcription factor FOXP3 (*FOXP3*). FOXP3 is a member of the forkhead box P (FOXP) family of transcription factors and is fundamental to the function of Tregs. Most affected children have failure to thrive. Additionally, patients with IPEX may have immune-mediated cytopenias, other manifestations of autoimmunity, severe food allergies, nephritis, and exaggerated responses to infections. Since manifestations are due to immune dysregulation, immunosuppression in the mainstay of treatment for IPEX while hematopoietic cell transplantation is the only curative therapy.

Abnormal T-regulatory (Treg) cells are thought to underlie the pathogenesis of several known autoimmune diseases through loss of self-tolerance. There is evidence for such abnormal function in relapsing-remitting multiple sclerosis (RRMS) [131], type 1 diabetes (T1D) [132], psoriasis [133], and immune thrombocytopenia (ITP) [134]. Moreover, Tregs also contribute to human diseases that involve inflammation, notably cancer and infectious diseases [135]. In addition, modification of Tregs has been proposed as immune therapy for autoimmune diseases, cancer, infectious diseases, and for achieving tolerance to transplanted organs [136].

Summary

Each leukocyte subpopulation – neutrophils, eosinophils, basophils, monocytes, and lymphocytes – has a critical role in host defense against pathogenic organisms. Some of the roles overlap, and others are unique to each class of cells. The members of each series exhibit variations in number or function in response to disease or infection. New data have emerged implicating neutrophils in the pathogenesis of autoimmunity and venous thrombosis. An understanding of the regulation and functions of each of these cells aids in understanding the clinical picture of a patient. Although the information provided here appears exhaustive, a considerable amount about the function of each of these cells has yet to be uncovered.

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Pediatric Hematology

20

Sanjay P. Ahuja

Introduction

Pediatric hematology has contributed much of the pioneering work in the basic understanding of hematopoiesis, granulocyte, and platelet function.

Development of Hematopoiesis

1. Development of the human hematopoietic system begins in the embryo. The erythrocytes produced by the human fetus are different from the erythrocytes of an older child. Fetal erythrocytes have different hemoglobins, different membrane properties, and a significantly shorter life span. These differences are necessary for the fetus to adapt to intra- and extrauterine life. In various pathologic states, erythrocytes bearing some of the properties of the fetal erythrocyte appear in the circulation.
2. Embryonic and fetal hematopoiesis can be divided into three periods: mesoblastic, hepatic, and myeloid. The mesoblastic period is predominantly in the yolk sac during the first trimester. The hepatic period predominates during the second trimester. The myeloid period, where the bone marrow takes over hematopoiesis, predominates during the third trimester.
   A. All blood cells are derived from the mesenchyme or the embryonic connective tissue. The earliest signs of blood formation can be detected by the 14th day of gestation. The first blood cells produced belong to the red cell series. They arise as a result of either the primitive megaloblastic erythropoiesis or the definitive normoblastic erythropoiesis. By the 10th week of gestation, normoblastic erythropoiesis is responsible for 90% of the erythrocytic cells. Hematopoiesis in the yolk sac or the mesoblastic period typically ceases by 10–12 weeks of gestation.
   B. Blood formation begins in the liver by the fifth to sixth week of gestation. From the third to the sixth fetal month, the liver becomes the chief organ of hematopoiesis. During this period, hematopoietic precursors can also be found in the spleen, the thymus, and the lymph nodes.
   C. Medullary hematopoiesis begins during the fourth to fifth fetal month. By the 30th gestational week, the marrow cavity is maximally cellular. The volume of the marrow cavity occupied by hematopoietic activity though continues to increase until term. During the last 3 months, the bone marrow is the predominant site of blood formation, whereas the hepatic site continues to decline.
   D. At birth, marrow tissue continues to grow, though the cellularity does not. Any increase in cell production during times of stress then happens with an expansion of hematopoietic tissue outside of the marrow giving rise to "extramedullary hematopoiesis," as seen in severe erythroblastosis fetalis or hemolytic disease of the newborn in the form of hepatosplenomegaly or in the form of "hemolytic facies," distortion of facial bones (frontal bossing, maxillary prominence), seen in β-thalassemia major and sickle cell anemia.
   E. Umbilical cord blood is rich in bone marrow progenitor cells and contains multipotential stem cells. The number of hematopoietic progenitors in cord blood is sufficient for a successful bone marrow transplant in children and adults. Human umbilical cord blood has been successfully used for hematopoietic reconstitution in patients with malignancies of the hematologic system, storage disorders, Fanconi's anemia or other bone marrow failure syndromes, as well as several immunodeficiencies.

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Hemoglobin Synthesis in the Fetus and the Newborn

1. The major form of adult hemoglobin (Hb) is Hb A1 ($\alpha_2\beta_2$), whereas in the embryonic and fetal stages, different forms of hemoglobins exist.
   A. Within 2 weeks of gestation, primitive erythroblasts in the yolk sac are already synthesizing the most primitive Hb, Gower 1 {$\zeta(\text{zeta})_2$, $\text{epsilon}(\varepsilon)_2$}.
   B. Synthesis of the α- and γ-chains begins in the yolk sac by 4–8 weeks of gestation. Synthesis of the ζ- and ε-chains decreases as that of the α- and γ-chains increases.
   C. By 6 weeks of gestation, Hb F ($\alpha_2\gamma_2$) has become the major Hb of fetal life. By the time the fetus has a crown-rump length of about 30 mm, Hb F represents 50% of the total Hb, and at a length of 50 mm, it forms more than 90% of the hemoglobin.
   D. At birth, term infants typically have 50–95% Hb F and the remainder Hb A.
   E. By 6 months to a year of life, Hb F declines to 1–2% and then persists at those levels throughout life.
2. The switch from fetal to adult Hb is delayed in infants who are small for gestational age, have chronic intrauterine hypoxia, or have been born to diabetic mothers.

Erythropoiesis After Birth

1. At birth, tissue oxygen levels increase, resulting in a marked decrease in the level of erythropoietin in the plasma. Production of red blood cells and hemoglobin decreases during the 1st week of life. In healthy term infants, no measurable decrease in hemoglobin values occurs during the 1st week of life. Term infants develop physiologic anemia by 8–10 weeks of life with a nadir Hb of 11 g/dL, whereas preterm infants nadir at 4–6 weeks with a Hb of 9–10 g/dL (Table 20.1).

   The life span of RBCs in term infants is shorter than that of the red cells of an adult. The life span is 60–70 days in term infants and 35–50 days in premature infants, whereas it is 120 days in an adult. Neonatal red cells are typically macrocytic with a mean MCV of 100 fl. The neonatal RBC also has lower cell hemoglobin (MCHC) concentrations than do adult RBCs. The macrocytosis decreases as age progresses reaching adult levels by 1 year of age (Table 20.1). Premature infants have more prominent macrocytosis probably as a result of immature splenic function.
2. The neonatal red cell membrane is slightly more resistant to osmotic lysis than those of adults. About 14% of the red cells from neonates show morphologic distortions such as spherocytes and poikilocytes of various types,

Table 20.1 Normal data have been compiled from children at various age groups

	Hemoglobin (g/dL)		Hematocrit (%)		Red cell count (10^{12}/L)		MCV (fL)		MCH (pg)		MCHC (g/dL)	
Age	Mean	−2 SD	Mean	−2 SD	Mean	−2 SD	Mean	−2 SD	Mean	−2 SD	Mean	−2 SD
Birth (cord blood)	16.5	13.5	51	42	4.7	3.9	108	98	34	31	33	30
1–3 days (capillary)	18.5	14.5	56	45	5.3	4.0	108	95	34	31	33	29
1 week	17.5	13.5	54	42	5.1	3.9	107	88	34	28	33	28
2 weeks	16.5	12.5	51	39	4.9	3.6	105	86	34	28	33	28
1 month	14.0	10.0	43	31	4.2	3.0	104	85	34	28	33	29
2 months	11.5	9.0	35	28	3.8	2.7	96	77	30	26	33	29
3–6 months	11.5	9.5	35	29	3.8	3.1	91	74	30	25	33	30
0.5–2 years	12.0	10.5	36	33	4.5	3.7	78	70	27	23	33	30
2–6 years	12.5	11.5	37	34	4.6	3.9	81	75	27	24	34	31
6–12 years	13.5	11.5	40	35	4.6	4.0	86	77	29	25	34	31
12–18 years Female	14.0	12.0	41	36	4.6	4.1	90	78	30	25	34	31
Male	14.5	13.0	43	37	4.9	4.5	88	78	30	25	34	31
18–49 years Female	14.0	12.0	41	36	4.6	4.0	90	80	30	26	34	31
Male	15.5	13.5	47	41	5.2	4.5	90	80	30	26	34	31

From Nathan and Stuart [6]

These data have been assembled from several sources. Emphasis is given to studies employing electronic counters and the selection of populations that are likely to exclude individuals with iron deficiency. The mean + SD can be expected to include 95% of the observation in the normal population

whereas this percentage is only 3% in an adult. The RBC membrane is also different from an immunologic perspective. In the ABO system, the A and B antigen sites are weakly expressed on the neonatal red cell membrane, and in the Ii antigen system, the I antigen is either weak or absent. The Ii RBC system is a minor antigen in contrast to the major ABO and Rh.

3. Hemoglobin and hematocrit values in neonates differ based on the site of sampling. Capillary samples obtained by a heel or toe stick generally have a higher hemoglobin concentration than do simultaneously obtained venous samples. The average Hb difference between the two methods is approximately 3.5 g/dl but could be as high as 10 g/dl. In virtually all infants, the capillary/venous hematocrit ratio is also greater than 1 and gradually decreases with increasing gestational age.

Myelopoiesis in Infancy and Childhood

1. The number of circulating leukocytes as well as neutrophils is elevated after birth in both full-term and premature infants. The mean WBC count at birth is 18.1 × 10^3/μL with 61% neutrophils and less than 15% bands. By the first month of life, mean WBC count is 10.8 × 10^3/μL, with 35% neutrophils and 56% lymphocytes. The relative lymphocytosis persists until 4–6 years of age. By age 6 years, WBC count declines to a mean value of 8.5 × 10^3/μL, with 53% neutrophils and 39% lymphocytes – adult values (Table 20.2).
2. The lower limit for normal neutrophil counts (neutrophil and band cells) is 1000 cells/μL in Caucasian infants between 2 weeks and 1 year of age. After infancy, the corresponding value is 1500 cells/μL. In African-Americans, the neutrophil counts are about 200–600 cells/μL lower relative to counts in Caucasians.

Platelets in Infancy and Childhood

1. Platelet count at birth may vary between 100 and 300 × 10^3/μL. Any platelet count less than 100 × 10^3/μL is considered abnormal for full-term as well as preterm infants.
2. After infancy, the normal platelet count of 150–400 × 10^3/μL is similar to that of adults.

Coagulation System in Infancy and Childhood

1. The development of the hemostatic system is a dynamic process which is age dependent. The neonate manifests physiologic alterations of this developing hemostatic system. Therefore, normal values of the coagulation protein factors are unique in this population by virtue of changes that occur from prenatal period to about 6 months of age.
2. A number of factors are responsible for the alterations of the hemostatic system seen at birth and for the few months after. There are decreased hepatic synthesis of coagulation factors, increased rates of clearance, and the synthesis of

Table 20.2 Reference ranges for leukocyte counts in children

	Total leukocytes		Neutrophils			Lymphocytes			Monocytes		Eosinophils	
Age	Mean	Range	Mean	Range	%	Mean	Range	%	Mean	%	Mean	%
Birth	18.1	9.0–30.0	11.0	6.0–26.0	61	5.5	2.0–11.0	31	1.1	6	0.4	2
12 h	22.8	13.0–38.0	15.5	6.0–28.0	68	5.5	2.0–11.0	24	1.2	5	0.5	2
24 h	18.9	9.4–34.0	11.5	5.0–21.0	61	5.8	2.0–11.5	31	1.1	6	0.5	2
1 week	12.2	5.0–21.0	5.5	1.5–10.0	45	5.0	2.0–17.0	41	1.1	9	0.5	4
2 weeks	11.4	5.0–20.0	4.5	1.0–9.5	40	5.5	2.0–17.0	48	1.0	9	0.4	3
1 month	10.8	5.0–19.5	3.8	1.0–9.0	35	6.0	2.5–16.5	56	0.7	7	0.3	3
6 months	11.9	5.0–17.5	3.8	1.0–8.5	32	7.3	4.0–13.5	61	0.6	5	0.3	3
1 year	11.4	6.0–17.5	3.5	1.5–8.5	31	7.0	4.0–10.5	61	0.6	5	0.3	3
2 years	10.6	6.0–17.0	3.5	1.5–8.5	33	6.3	3.0–9.5	59	0.5	5	0.3	3
4 years	9.1	5.5–15.5	3.8	1.5–8.5	42	4.5	2.0–8.0	50	0.5	5	0.3	3
6 years	8.5	5.0–14.5	4.3	1.5–8.0	51	3.5	1.5–7.0	42	0.4	5	0.2	3
8 years	8.3	4.5–13.5	4.4	1.5–8.0	53	3.3	1.5–6.8	39	0.4	4	0.2	2
10 years	8.1	4.5–13.	4.4	1.8–8.0	54	3.1	1.5–6.5	38	0.4	4	0.2	2
16 years	7.8	4.5–13.0	4.4	1.8–8.0	57	2.8	1.2–5.2	35	0.4	5	0.2	3
21 years	7.4	4.5–11.0	4.4	1.8–7.7	59	2.5	1.0–4.8	34	0.3	4	0.2	3

From Nathan and Orkin [6]

Numbers of leukocytes are in thousands per mm³, ranges are estimates of 95% confidence limits, and percentages refer to differential counts. Neutrophils include band cells at all ages and a small number of metamyelocytes and myelocytes in the first few days of life

Table 20.3 Reference values for coagulation tests in healthy, full-term newborns compared with normal adults

Test	Newborns	Adults	*P*<
PT (s)	13.1 ± 0.9	11.9 ± 0.6	0.0001
aPTT (s)	35 ± 4.5	28.8 ± 2.7	0.0001
Platelets ($\times 10^9$/L)	214 ± 55	258 ± 66	0.0001
Fibrinogen (mg/dL)	251 ± 51	262 ± 44	NS
Factor II (%)	73 ± 7	100 ± 15	0.0001
Factor V (%)	93 ± 13	98 ± 19	NS
Factor VII (%)	88 ± 12	95 ± 18	0.005
Factor VIII (%)	113 ± 38	92 ± 21	0.0001
Factor IX (%)	86 ± 18	94 ± 16	0.003
Factor X (%)	72 ± 10	97 ± 15	0.0001
Hematocrit (%)	59 ± 3.0	44 ± 2.5	0.0001

From Nathan and Orkin [6]
Data were obtained in 71 newborns and 100 adults and expressed as mean ± SD. Samples were collected with a constant anticoagulant-to-blood ratio, based on a previous determination of hematocrit
NS Not Significant

fetal forms of many factors. For this reason, age-appropriate reference ranges are necessary to adequately interpret coagulation results in preterm and term neonates (Table 20.3).

3. Despite striking differences in the levels of individual components of the hemostatic system, the neonatal coagulation is equal to or somewhat more robust than that observed in adults. The balance of the coagulation system is more weighted toward hypercoagulability and potential thrombosis in the sick infant.
4. Coagulation proteins are synthesized by the fetus and present in measurable quantities by approximately 10 weeks of gestational age. Values of vitamin K-dependent factors, prekallikrein, factor XII, high molecular weight kininogen, and fibrinolytic and anticoagulant factors typically normalize by 6 months of age.
   A. PT and aPTT are usually prolonged in the neonatal period secondary to physiologic deficiency of vitamin K-dependent factors at birth. However, the thrombin time is normal.
   B. Concentrations of fibrinogen, FV, and FXIII are similar to adult values at birth. In contrast, levels of FVIII and von Willebrand factor (vWF) are increased in the neonatal period. The mean activity of several factors (II, VII, IX, X, XI, XII) is slightly lower throughout childhood (Table 20.3).
   C. Plasma concentrations of antithrombin and heparin cofactor II are decreased at birth, whereas plasma concentrations of α_2 macroglobulin are increased at birth and throughout childhood. Plasma concentrations of protein C are decreased at birth, with levels usually less than those reported for heterozygote-deficient adults. Although total amounts of protein S are decreased at birth, the overall activity remains similar to adults. Levels of protein C do not reach adult range until puberty.

Disorders of Fetomaternal Unit Causing Hematologic Manifestations in the Neonate

1. *Placental Insufficiency*: The most common causes of placental insufficiency resulting in intrauterine growth retardation (IUGR) are pregnancy-induced hypertension, preeclampsia, eclampsia, and the HELLP (*h*emolysis, *e*levated *l*iver enzymes, and *l*ow *p*latelets) syndrome. Disruption of placenta causes hematopoietic dysfunction, and its severity is proportional to the degree of placental insufficiency and fetal growth restriction.
   A. Mild placental dysfunction with tissue hypoxia is associated with increased erythropoietin levels and polycythemia. Severe placental vasculopathy is seen in babies with severe IUGR. This vasculopathy causes decreased erythropoiesis due to abnormal erythroblast function leading to fetal anemia. RBC destruction from microangiopathic injury in the placenta is an additional mechanism of fetal anemia.
   B. The normal nucleated RBC (nRBC) count in healthy term neonates is variable but rarely exceeds 8 nRBCs/100 white blood cells. An elevated nRBC count is associated with placental insufficiency and fetal stress, though it is a nonspecific marker and can be seen in other forms of fetal/neonatal stress such as Rh isoimmunization (hemolytic disease of the newborn), fetal hemorrhage, maternal diabetes mellitus, and respiratory distress syndrome (RDS).
   C. About 40–50% of neonates born to mothers with placental insufficiency are neutropenic; the cause is decreased neutrophil production due to a placental inhibitor present in cord blood. Even though neutrophil counts below 500/μL are frequently seen, there does not appear to be a significant risk of infection. The neutropenia usually resolves in less than 72 h, but can last up to 5 days.
   D. Growth-restricted neonates born to mothers with placental insufficiency can also have thrombocytopenia, especially if the mother has HELLP syndrome. This phenomenon is more common in preterm infants and is caused by decreased platelet production.
2. *Abnormal Fetomaternal Cell Traffic*: Clinically important fetomaternal transfusions occur in up to 50% of all pregnancies, and some fetal cells can be demonstrated in the maternal circulation. Risk factors for fetomaternal transfusion or hemorrhage include abdominal trauma, amniocentesis, external cephalic version (a manual procedure to allow for breech baby to have vaginal delivery), and placental tumors. They can present as neonatal anemia when associated with chronic blood loss or as fetal hypotension and intrauterine demise when associated with acute blood loss. Fetomaternal transfusions can also result in alloimmunization of the mother against paternal antigens present on fetal blood cells. The placenta is a "leaky" barrier as it serves an important nutrient function for the

fetus besides providing transfer of passive immunity. IgG antibodies are able to cross over into the fetus, but IgM antibodies cannot. The transplacental transfer of these maternal antibodies into the fetus can cause destruction of the fetal blood cells carrying the cognate antigen. Hematologic consequences of this abnormal antibody traffic are several.

A. *Hemolytic Disease of the Newborn (HDN, Erythroblastosis Fetalis)*

Overview. HDN is classically seen in a setting of blood group incompatibility between the mother and her fetus for markers of the Rh system, which includes five antigens: C, D, E, c, and e. HDN is also seen with ABO or minor blood group incompatibilities between the mother and her fetus. Rh-mediated HDN affects about 1 in 1200 pregnancies. In approximately 97% of cases, erythroblastosis fetalis is caused by anti-Rh(D) antibody, with the remaining 3% of cases caused by a variety of other antibodies to less common RBC groups.

Pathophysiology. Passage of fetal Rh(D)-positive RBCs across the placental barrier to a Rh-negative mother is the primary method of maternal alloimmunization. Fetomaternal bleeding at the time of delivery is the major cause of sensitization in Rh-mediated HDN. About 10–15% of Rh-negative women are sensitized while bearing children of Rh-positive men. The disparity between the numbers of incompatible and alloimmunized pairs is due to:

1. Threshold effect in fetomaternal transfusions, where a certain amount of the immunizing blood cell antigen is required to activate the immune system: in ABO incompatibility, transfer of 0.1 mL of blood leads to sensitization in 3% of women as compared to 60% of women with transfer of 5 mL of blood.
2. Type of antibody response: IgG antibodies are more efficiently transferred across the placenta to the fetus. In Rh-mediated HDN, IgM antibodies are produced initially which are replaced by IgG antibodies. In future pregnancies, these IgG antibodies can cross the placenta easily causing more severe disease as the number of pregnancies rises. Rh-related HDN is therefore usually a disease of the second and subsequent pregnancies.
3. Differential immunogenicity of blood group antigens: hemolysis due to anti-A is more common (1 in 150 births) than due to anti-B antibodies in ABO HDN.
4. Differences in maternal immune response: even though 15–25% of all maternal/fetal pairs are ABO-incompatible, ABO HDN occurs in about 1% of these pairs that have preexisting high-titer IgG antibodies.

Clinical Features

1. Anemia, mild to severe resulting from destruction of antibody-coated RBCs, mainly in the spleen. Severe anemia can lead to cardiac dilatation, hypertrophy, and subsequent high-output failure and ultimately hydrops fetalis, a dead fetus due to severe intrauterine anemia.
2. Jaundice (indirect hyperbilirubinemia) presenting during the first 24 h. It may cause kernicterus, resulting from bilirubin deposition in the developing brain leading to reduced intellectual development.
3. Hepatosplenomegaly, as a result of compensatory extramedullary hematopoiesis.
4. Hypoalbuminemia and hepatic dysfunction leading to generalized edema, hydrops, and ultimately intrauterine death. Depending upon severity, stillbirth or delivery of a macerated fetus is also possible.
5. Petechiae in severely affected infants. Hyporegenerative thrombocytopenia and neutropenia may occur during the 1st week.

Laboratory Findings

1. Anemia with increased reticulocyte count
2. Direct Coombs' test positive on fetal RBCs
3. Increased nucleated RBCs, marked polychromasia, and anisocytosis on peripheral smear
4. Raised indirect serum bilirubin concentration

Management and Prevention

1. Both planned, careful prenatal monitoring and therapeutic intervention are needed for prevention and successful treatment of HDN. Mothers should be screened at their first antenatal visit for Rh and non-Rh antibodies. Rh-negative women have to undergo determination of anti-Rh antibody titers to determine the degree of sensitization. Any titers above 1:16 or 1:32 are considered high risk for development of hydrops fetalis. Father's blood is tested for Rh zygosity.
2. Examination of amniotic fluid for spectrophotometric analysis of bilirubin is done to determine the most suitable time for delivery or to determine the indication for intrauterine fetal transfusion. Amniocentesis is indicated if (a) there is history of previous Rh disease severe enough to require an exchange transfusion or which has caused stillbirth and (b) maternal titers of antibodies are between 1:8 and 1:64 or greater by indirect Coombs' test. Intensive maternal plasmapheresis antenatally using a continuous-flow cell separator can significantly reduce Rh antibody levels, reduce fetal hemolysis, and improve fetal survival in those mothers carrying highly sensitized Rh-positive fetuses.
3. Determination of fetal biophysical profile score, a criteria standard to determine if intrauterine intravascular transfusion (IUIVT) should be performed, is done with the help of optical density of the amniotic fluid, ultrasound to assess hydrops, and amniotic phospholipid determination done to assess lung maturity. The fetal biophysical profile is a test scoring of five fetal vital sign variables: fetal heart rate, fetal breathing, fetal movement, fetal tone, and amniotic fluid volume. If the biophysical profile estimates a severely affected fetus and the phospholipid estimations indicate marked

immaturity, IUIVT should be carried out. IUIVT is the procedure of choice as compared to intraperitoneal transfusions.

4. The IUIVT procedure involves high-resolution ultrasound-guided fine needle insertion directly into the umbilical cord to gain access to the intravascular space. Compatible Rh-negative RBCs are used for IUIVT. The risk of fetal loss with an IUIVT is about 2%.
5. Modern neonatal care with rigorous attention to metabolic, ventilatory, and nutritional needs and use of artificial surfactant delivered via inhalation have made successful deliveries possible with severe HDNs. The need for IUIVT and intraperitoneal transfusion is rarely, if ever, indicated.
6. Postnatally, the most frequent problem is hyperbilirubinemia. The umbilical cord blood is examined for Coombs' test, bilirubin, and Hb levels. A rapid increase in the bilirubin level of greater than 1.0 mg/dL/h and/or a bilirubin level approaching 20 mg/dL at any time in a full-term infant and 15 mg/dl in a preterm infant is an indication for exchange transfusion to prevent development of kernicterus. An exchange transfusion is done using 10–20 mL aliquots of group O (or ABO compatible) Rh-negative blood to replace twice the infant's blood volume. This procedure removes 50% of the anti-Rh antibody and can be repeated to keep the bilirubin below 20 mg/dL. A partial exchange transfusion or a double-volume exchange transfusion may be necessary to correct severe anemia in a hydropic infant at birth.
7. To prevent Rh-mediated HDN, routine administration of 300 μg of Rh immunoglobulin to all unsensitized Rh-negative mothers is recommended at 28 weeks of gestation and within 72 h of delivery. It is also indicated for all unimmunized Rh-negative mothers who have undergone spontaneous or induced abortion, or a ruptured tubal pregnancy, or any event during pregnancy that may lead to transplacental hemorrhage. Antibody to Rh is infused to block any Rh-positive blood that may have gotten into the maternal circulation and thus rear antibodies to the Rh antigen.

B. *Alloimmune Neonatal Neutropenia*

1. Alloimmune neonatal neutropenia (ANN) is the neutrophil counterpart of HDN. Mother makes antibodies against paternal antigens (also present in the fetus) which cross the placenta and cause neutropenia in the fetus. These antibodies can be detected in up to 20% of pregnant and postpartum women, but ANN occurs in only 0.2–2% of newborns. In the USA, almost half of all cases of ANN are mediated by antibodies to HNA-1a, HNA-1b, or HNA-2a antigens.
2. Common presentations of ANN include delayed separation of the umbilical cord, mild skin infections, fever, or pneumonia within the first 2 weeks of life. ANN can last up to 6 months, though it usually resolves by 4–6 weeks. Recombinant human granulocyte colony-stimulating factor (G-CSF) may be indicated for prolonged, severe neutropenia (<500/μL) or a severe infection.

C. *Neonatal Alloimmune Thrombocytopenia*

1. Neonatal alloimmune thrombocytopenia (NAIT) is the platelet counterpart of HDN. NAIT occurs in approximately 1 in 2000 live births. Maternal antibodies directed against fetal platelet antigens lead to increased platelet destruction. The commonest antigens causing fetomaternal incompatibility in almost 95% of the cases are HPA-1a, HPA-5b, and HPA-15b either singly or in combination.
2. It is characterized by marked thrombocytopenia in the fetus and the neonate with platelet counts as low as $10 \times 10^3/\mu L$. Infants affected with NAIT are born to mothers with a normal platelet count as compared to infants born with ITP (NITP) to mothers with immune thrombocytopenia.
3. NAIT is associated with mortality of about 15%, mostly due to the occurrence of intracranial hemorrhage which can occur even in utero. Daily ultrasound of the newborn's brain should be carried out during the acute phase to detect intracranial hemorrhage. Bleeding from the umbilicus or gastrointestinal or renal tract may also occur.
4. Treatment depends on the platelet count and symptoms and can include transfusion of random donor platelets or HPA-1a-negative platelets or maternal platelets. The antigen system HPA-1a accounts for approximately 75% of the cases of NAIT. As maternal antibodies against fetal HPA-1a antigen are causing platelet destruction, transfusion of HPA-1a-negative platelets can help stop this destruction and increase platelet count. IVIG and/or corticosteroids can also be used to reduce platelet destruction. IVIG flood the reticuloendothelial cell system with immunoglobulin, reducing clearance of antibody-coated RBCs. Weekly IVIG may also be used antenatally for the mother from midgestation until birth. Unfortunately, antiplatelet antibody titers in the mother during pregnancy cannot be used to predict whether an individual fetus will be affected.

3. *Transplacental Transfer of Maternal Autoantibodies*: Transplacentally transmitted maternal autoantibodies against RBC, neutrophil, and platelet antigens can cause destruction of one or more of these cell lines in the fetus. Autoantibodies against hematopoietic lineages are

frequently of the IgG1 subclass, which are transmitted to the fetus with high efficiency.

A. *Neonatal autoimmune hemolytic anemia* is seen in infants born to mothers with autoimmune hemolytic anemia, SLE, rheumatoid arthritis, or other immune disorders. It is a self-limited condition and resolves within a few weeks to months. It is diagnosed by a positive Coombs' test in the mother and the infant. Treatment with steroids may be needed to decrease the severity of the illness.
B. *Neonatal autoimmune neutropenia* is caused by transplacental transmission of maternal antineutrophil autoantibodies. Both the mom and the baby are neutropenic. It is an asymptomatic condition, and treatment with IVIG and/or G-CSF may be needed only in severe cases or in infants with an active infection.
C. *Neonatal autoimmune thrombocytopenia* (NITP) is milder than NAIT and carries a low risk (<1%) of intracranial hemorrhage or bleeding at other sites. It is seen in 10% of infants born to mothers with ITP, SLE, or other immune disorders. Symptomatic infants can be effectively treated with IVIG.

Further Reading

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Bone Marrow Structure and Marrow Aspiration, Biopsy, and Collection for Therapeutic Intent Procedures

21

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Bone Marrow Structure

A. *Overview.* The bone marrow is a semisolid gelatinous tissue which resides within the bony cavities of the axial skeleton [1–7]. It contains hematopoietic cells (red marrow), stromal cells, and fat (yellow marrow).

B. *Bone*. The bone that surrounds the marrow is composed of a thick layer of compact material referred to as the *cortical bone*. This bone is covered by a fibrocartilaginous tissue (*periosteum*). The space inside the cortical bone is called the *medullary cavity*. The medullary cavity itself contains a lattice-like network of thin bone referred to as *trabecular* or *cancellous* bone. Hematopoietic cells reside in the intervening spaces of trabecular bone (Fig. 21.1).

1. *Structure of the bone*
   (a) *Woven bone* is tissue in which the normal parallel fibrillar structure of the bone has not been fully created. It is the pattern seen in newly formed bone.
   (b) *Lamellar bone* is a mature bone in which there is a microscopically visible parallel structure referred to as *cement lines*.
2. *Cellular components of the bone*
   (a) *Osteoblasts*
      1. Osteoblasts are bone-forming cells of mesenchymal origin that synthesize glycosaminoglycans and collagen fibers (*osteoid* or non-mineralized bone) forming the basis of bone structure.
      2. On tissue biopsy sections, the cells are flat to slightly rounded and line the bony trabeculae and cortical bone particularly in areas of new bone formation (Fig. 21.1).
      3. On aspirate smears the cells resemble plasma cells (although much larger) with blue cytoplasm, a distinct Golgi apparatus and an eccentric nucleus although the nucleus of an osteoblast often appears to be falling out of the cell. The cells commonly occur in small loose collections (Fig. 21.2).
      4. Osteoblasts are not generally encountered in bone marrow aspirated from normal adults but may be seen in growing children, sites of bony injury, or hyperparathyroidism.
   (b) *Osteoclasts*
      1. Osteoclasts are bone resorbing/remodeling multinucleated cells derived from the monocyte/macrophage lineage.
      2. The cells reside in scooped out areas of trabecular bone referred to as *Howship's lacunae*.
      3. Osteoclasts, like osteoblasts, are not normally encountered on an aspirated bone marrow smear unless bone remodeling changes are present or bone growth is occurring.
      4. Osteoclasts are very large cells up to 100–150 μm in size and resemble megakaryocytes due to their size and multi-nucleation containing 5–20 nuclei. The cells differ from megakaryocytes as their nuclei are separate and distinct rather than overlapping as seen in the megakaryocyte (Fig. 21.3).
3. *Biology of the bone*
   (a) The skeleton undergoes continuous remodeling during life. The remodeling is carried out by

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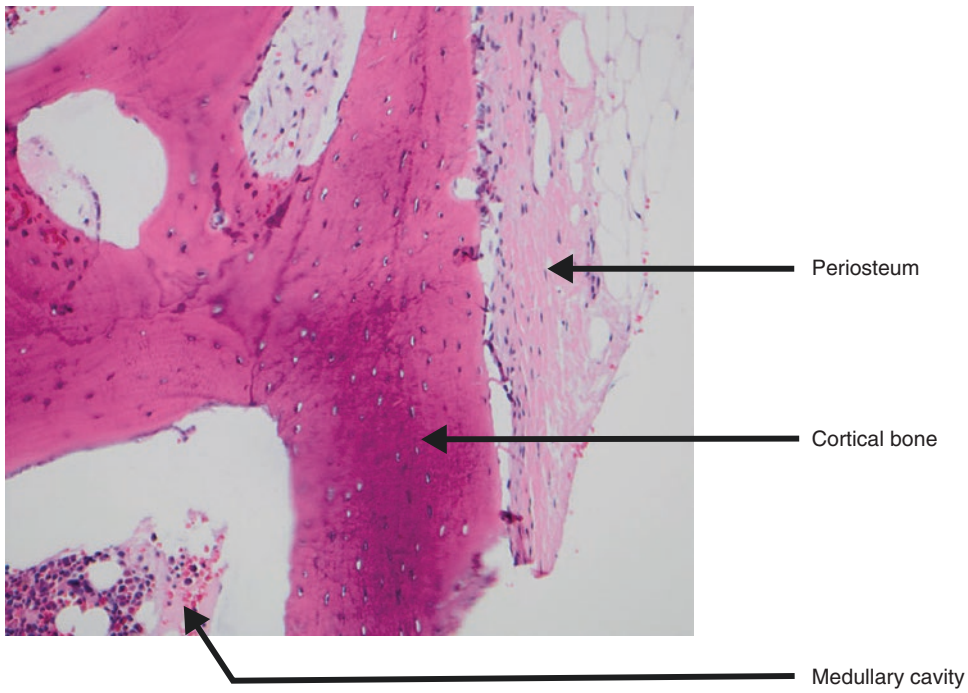

Fig. 21.1 Bone marrow core biopsy, 40×

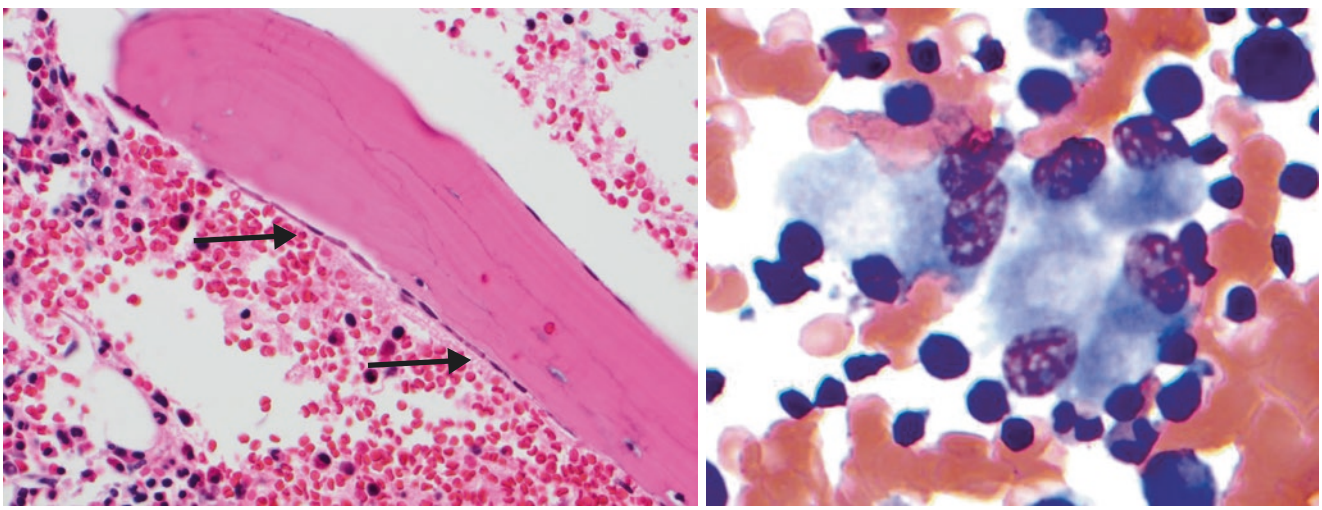

Fig. 21.2 Osteoblasts on bone marrow core biopsy (400×), left, and bone marrow aspirate smear (1000×), right

osteoclasts resorbing bone followed by osteoblasts producing osteoid, which then becomes mineralized.

(b) Osteoblastic and osteoclastic activity is coupled together.
   1. Osteoclastic activity is stimulated by the action of osteoblasts which are activated by various environmental signals.
   2. Bone resorption by osteoclasts requires *r*eceptor *a*ctivator of *n*uclear factor *k*appa-B *l*igand (*RANKL*), a member of the TNF ligand protein family expressed by osteoblasts. RANKL stimulates osteoclastic differentiation, cellular fusion generating multinucleated cells, and activation resulting in bone resorption [8].
   3. Osteoclasts subsequently direct the differentiation and activation of osteoblasts to synthesize new bone through the action of both membrane-bound and secreted signaling molecules.

(c) *Osteocytes* are cells that are encased by bone and appear as small nuclei within trabecular or cortical bone. They are terminally differentiated osteoblasts.

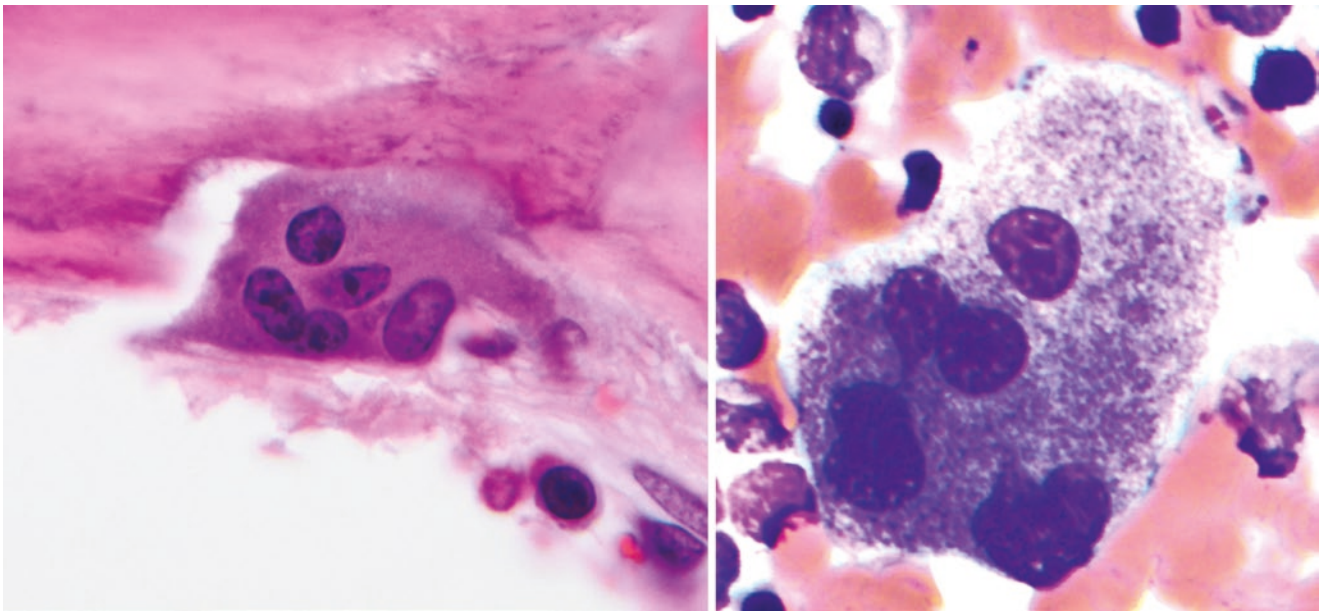

Fig. 21.3 Osteoclast on bone marrow core biopsy (1000×), left, and bone marrow aspirate smear (1000×), right

C. *Components of the bone marrow*
1. *Bone marrow stroma*
   (a) The bone marrow stroma is the supporting matrix for hematopoietic cells.
   (b) The bone marrow stroma is composed primarily of:
      1. Macrophages
      2. Specialized bone marrow stromal cells
         (a) Bone marrow stromal cells have the important ability to differentiate into other mesenchymal tissues such as bone, cartilage, and fat.
         (b) Stromal cells also provide important micro-anatomical niches supporting hematopoiesis and hematopoietic stem cells.
         (c) Bone-differentiated stromal cells comprise a distinct niche that supports B-lymphoid progenitors and stromal cells in the perivascular region support other hematopoietic stem cells.
         (d) Stromal cells support hematopoietic stem cells via expression of C-X-C motif chemokine 12 (CXCL-12), also known as stromal cell-derived factor 1 (SDF-1), through its interaction with C-X-C chemokine receptor type 4 (CXCR4).
      3. Fat cells (adipose tissue)
         (a) The function of marrow adipose tissue in regulating hematopoiesis or osteogenesis is unclear. Marrow fat secretes cytokines that can influence granulopoiesis and T cell and monocyte function.
         (b) There is an inverse relationship between amount of marrow fat and erythropoiesis and bone density not seen with subcutaneous adipose tissue.
         (c) Marrow adipose tissue also differs from peripheral adipose tissue in that it is not lost in starvation.
2. *Vasculature*
   (a) The marrow vasculature consists of a *nutrient artery* that penetrates the bone and branches into smaller and smaller divisions (arterioles) ultimately forming open vascular channels, a *sinusoidal network*, in the medullary cavity.
   (b) Sinusoids are dilated thin-walled channels at the capillary-venous junction.
      1. Adventitial cells:
         (a) Sinusoids are lined on the outside by adventitial cells.
         (b) Adventitial cells serve as an additional niche supporting hematopoiesis.
         (c) Adventitial cells likely make *reticulin*, a form of collagen, which can increase in disease states.
      2. Morphologically, on tissue sections, sinusoids are generally not apparent since they are often collapsed.
      3. Sinusoids drain into venous sinuses which connect to ultimately form the *comitant* vein.

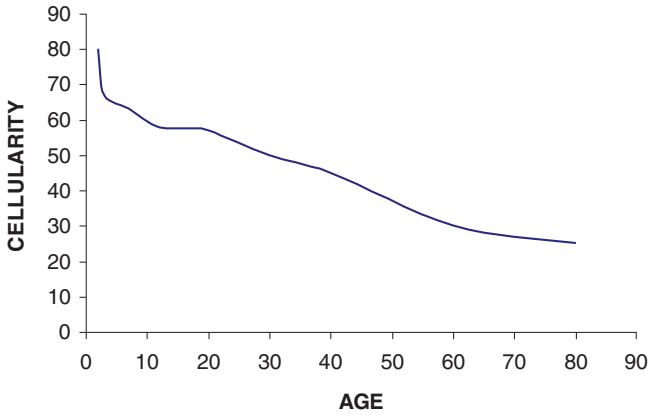

Fig. 21.4 Relationship of bone marrow cellularity with age

Table 21.1 Clinical reasons to perform a bone marrow examination

Unexplained cytopenia (anemia, thrombocytopenia, neutropenia)
Staging for non-Hodgkin or Hodgkin lymphoma
Suspected acute leukemia or recurrence of acute leukemia
Suspected myeloproliferative disorder
Suspected myelodysplastic syndrome
Suspected metastatic carcinoma
Suspected plasma cell neoplasm (monoclonal immunoglobulin present in serum)
Evaluation of lymphoproliferative disorder
Staging of small cell tumors of childhood
Follow-up and treatment response in patients with hematopoietic neoplasms
Assessment of bone marrow after hematopoietic stem cell transplantation
Unexplained leukoerythroblastic blood smear
Fever of unknown origin
Evaluation of suspected macrophage disorder (storage disease, hemophagocytosis)

(c) The comitant vein exits the marrow through the same channel as the nutrient artery.

3. *Hematopoietic cells*
   (a) Hematopoietic cells lie in groups or cords in the intertrabecular spaces and enter the circulation by migrating through the sinusoidal endothelium.
   (b) Hematopoietic cells are not randomly distributed through the marrow but are present in small niches. On histologic sections these zones are not always evident.
      1. Erythroid progenitors localize in small collections around marrow macrophages.
      2. Granulocytes generally reside adjacent to adipose cells with immature granulocytes near bony trabeculae and adventitial cells.
      3. Megakaryocytes lie abutting marrow sinusoids.
   (c) Marrow cellularity:
      1. The ratio of marrow space occupied by hematopoietic cells to the total available marrow space is referred to as the *marrow cellularity*.
      2. Marrow hematopoietic cellularity varies with age (Fig. 21.4). As a rule of thumb, marrow cellularity is equivalent to 100 minus the patient's age in years. In reality, marrow cellularity decreases to roughly 50% by age 30 and declines slowly thereafter until late adulthood.
      3. Marrow cellularity may change rapidly in response to a peripheral cytopenia, infection, or growth factors.

Bone Marrow Diagnostic Testing

A. *Overview*
Clinical reasons to evaluate the bone marrow are given in Table 21.1. In general a bone marrow examination should be done for an unexplained cytopenia, evaluation for a lymphoproliferative process, or suspected hematopoietic disorder. A thorough examination of a suspected marrow process consists of a review of the blood cell counts and indices and microscopic review of the peripheral blood smear. Medical history is important, and the reason for the marrow biopsy needs to be transmitted to the pathologist to help focus the evaluation. Bone marrow morphology changes dramatically in the face of chemotherapy. Therefore, the timing of the marrow biopsy in relation to therapy needs to be known. A bone marrow performed 14 days after initiating chemotherapy has a significantly different appearance than a marrow examined 35 days later. A history of recent treatment with hematopoietic growth factors is especially relevant.

B. *Bone marrow aspirate and biopsy procedure*
   1. *Overview.* The diagnostic bone marrow aspiration and biopsy procedure is an essential tool in the evaluation of patients who have a suspected hematologic disorder. This technique provides tissues that are analyzed by morphologic methods for cellularity and cellular characteristics, flow cytometry for immunophenotyping, and cytogenetic and fluorescent in situ hybridization (FISH) studies as well as for molecular markers. A variety of disorders can be diagnosed including bone marrow failure states, hematologic malignancies, metastatic cancers, enzymatic and congenital storage diseases, and other conditions.

      As with any invasive procedure, bone marrow aspiration and biopsy are best performed by trained clinicians who are aware not only of the indications and contraindications but also the prevention and management of potential complications. There are links on the web to videos that demonstrate the standard pro-

cedures for bone marrow aspiration and biopsy from the posterior superior iliac crest [9].

2. *Personnel and equipment*:
   (a) *Medical practitioner*. The bone marrow aspirate and biopsy procedure has been part of the stock-and-trade of the hematology-oncology physician for decades. With proper training, this technique can be undertaken successfully in most patients by medical practitioners such as internists, house officers in training and, depending on the state in which a person practices, mid-level care providers (licensed nurse practitioners and physician assistants). Procedures in very young children, however, should be restricted to individuals highly experienced in this patient subpopulation. In general, there are no absolute contraindications to performing this test. Caution, however, must be exercised in seriously ill patients or in those subjects with bleeding disorders (see below: Complications). Untrained medical personnel must be supervised extremely closely when performing this procedure and must not be permitted to undertake this testing without having fulfilled all local institutional policies and procedural regulations. Further, only very highly trained professionals should be performing diagnostic bone marrow examinations from the sternum due to the great danger of penetration into critical structures in the chest cavity; nearly all procedures should be undertaken at the posterior superior iliac crest site (see below).
   (b) *Additional personnel and necessary equipment*:
      1. *A trained laboratory assistant*. This person takes the specimens, prepares the slides for morphologic evaluation, and submits the bone marrow clot and biopsy in containers with appropriate media.
      2. *Chlorhexidine swabs* unless contraindicated due to a serious adverse reaction, in which case povidone-iodine can be used for sterilization purposes.
      3. *Sterile gloves.*
      4. *Prepacked bone marrow diagnostic kit* containing sterile drapes, ampules of local anesthetic (1% plain lidocaine or 0.25%/0.5% bupivacaine), disposable needles and syringes, and disposable sterile bone marrow aspiration and biopsy needles.
3. *Consenting process.* The patient must be identified as the appropriately intended person, and the subject (or guardian) must furnish consent for this procedure. Further, although extremely rare, the patient must be made aware of potential complications that have been reported to occur at a rate of 0.12–0.30% [10, 11]. Always verify that the patient is not allergic to either the local anesthetic or any other agents used for sedation.
4. *Complications*. In survey reports [10, 11] of 20,323 procedures, hemorrhage was the most frequent and most serious adverse event, necessitating rare blood transfusions and leading to death in a single patient. Potential risk factors most often associated with bleeding were a diagnosis of a myeloproliferative disorder, aspirin therapy, use of other anticoagulant treatment, disseminated intravascular coagulation, and obesity. Other complications have included trauma such as lacerations to tissues in the region, local infection, and hemorrhage. Other very rare, serious adverse events include retroperitoneal hematomas due to puncture of a branch of the iliac artery that runs on the inner surface of the hip [12, 13] and fractures of the bone that has been penetrated, especially in those individuals who have underlying bone disorders.
5. *Sedation.* Prior to the bone marrow aspirate and biopsy procedure, the patient may experience significant anxiety, and during and after the procedure, pain may be a prominent feature. The patient should be reassured that the test is indicated at the time this person is giving consent. Some patients may require use of minor tranquilizing or sedating agents. During the procedure itself, the patient should be given a judicious amount of local anesthesia but not exceeding local institutional guidelines. In those patients in whom their anxiety is excessive, possibly due to problems during a previous procedure, or in pediatric patients, conscious sedation administered by an anesthesiologist or other qualified health-care professional should be used.
6. *Preparation for the procedure:*
   (a) Site. One of the most important aspects of the procedure is identifying the appropriate site for aspirate and biopsy. The patient can be in the prone or ventral supine position or in the lateral decubitus position. Many physicians mark the posterior superior iliac crest with a marking pen and straw or use a coin to make an impression. The posterior superior iliac crest is lateral to the sacroiliac joint and can be palpated easily even in moderately obese patients. In more obese patients, identify the anterior iliac crest, and use the same latitude to identify the posterior superior iliac crest (see photograph).
   (b) Sterilization and anesthesia. The site is sterilized with chlorhexidine, and a sterile (windowed) drape is placed over the area prior to administering local anesthesia. Topical skin anesthesia can be used to number skin for injection of local anesthesia. An injection wheal is raised by injecting sub-

cutaneously with a 25 gage needle. Then, the anesthetic is infiltrated into the subcutaneous tissues by slowly advancing the needle to the periosteal tissues. Injecting the periosteal tissues is usually the most painful part of the procedure. Sufficient time should be made to allow for the local anesthetic to take effect.

7. *Bone marrow aspiration and biopsy:*
   (a) *Overview.* Many clinical personnel prefer to perform the aspirate and biopsy using the same instrument although two separate instruments can be used. Further, many persons prefer to enter the skin directly with the instrument rather than use a lance to create a small skin incision (and as such may leave a scar).
   (b) *Bone marrow aspiration:*
      1. Some bone marrow aspiration needles have a guard to limit the degree of penetration and prevent the needle from passing all the way through the bone. The guard is removed from the instrument except for sternal procedures (see above; use of sternal sites is to be discouraged except in very rare circumstances and only by highly qualified and experienced health-care providers).
      2. The angle of entry is important and the needle should be positioned so that at the start of insertion, the instrument is perpendicular to the posterior superior iliac crest prominence (see photograph).
      3. The needle is slowly advanced through the bone cortex using a semicircular motion with care to note the degree of penetration, usually only a few centimeters. Practitioners use different ways to define depth of insertion; one simple method is to put the index finger about 1 cm above the skin and stop advancing when this distance is achieved. Once inside the medullary area of the bone, the needle will feel fixed solidly within the bone.
      4. At this juncture, the stylet is removed, and a syringe is affixed to withdraw/aspirate approximately 0.5–1 mL of liquid bone marrow into the syringe. The syringe is removed and passed to the laboratory assistant to prepare the microscopic slides. An adequate marrow aspirate will have visible particles. If no particles have been obtained, reasons include either failure to adequately penetrate the bone marrow cavity, or the patient may have a hypoplastic or aplastic marrow space with limited or no hematopoietic tissue. In such instances, the person performing the procedure may withdraw the instrument and begin again using the same skin site but redirecting the needle in a slightly different position. Failure to obtain a marrow aspirate is referred to as a "dry tap."
      5. On occasion, additional samples for research are to be collected as well as for archiving material for later investigation. In order to provide materials representative of bone marrow (as opposed to peripheral blood), it is important to use the same (or different) skin puncture site but a different marrow location.
      6. The volume of marrow sample aspirated has very important consequences, especially while assessing response to therapy using techniques to assess minimal residual disease (MRD). The extent of MRD is a significant prognostic factor in predicting relapse after therapy in certain hematologic malignancies.

         Some investigators have shown that large volume marrow aspirates (above 3–5 mL) will result in a falsely lower blast counts as a consequence of hemodilution [14–17]. This situation can lead to an erroneous interpretation low MRD concentration and failure to upgrade therapy appropriately (see Chaps. 21, 22, and 23 on "Bone Marrow Flow Cytometry," "Cytogenetics," and "Molecular Diagnosis"). Hence bone marrow aspiration techniques should be standardized to limit the volume of aspirates to 0.5–2.5 mL.
   (c) *Bone marrow biopsy*:
      1. Usually the same skin site used for the aspirate is used for the biopsy, although the direction and bone insertion site may differ slightly. As noted above, many practitioners use the biopsy needle to collect both an aspirate and biopsy, separated in time.
      2. The instrument is inserted slowly into the bone using a semicircular motion of the hand but with gentle pressure. Bones may vary in composition from extremely hard to extremely soft, and caution is urged when initiating this maneuver. The needle is inserted into the bone until it appears fixed and stable (usually after about one-half to 1 cm of depth).
      3. The stylet is withdrawn, and the needle is slowly advanced a minimum of 1.5 cm using the semicircular motion.
      4. The needle is removed with the specimen in place. This is accomplished by gently rocking the needle back and forth in various directions while also twisting/rotating the needle clockwise and counterclockwise. Failure to initiate these actions often results in the bone marrow

biopsy (or "core") being left within the patient when the needle is removed.

5. Upon removal of the needle, the core is extracted from the sharp end of the needle by pushing a thin stylet into the needle until the biopsy has been pushed out of the proximal end (the contact point used for the syringe while performing the aspiration).
6. The biopsy or core should be pushed onto a small sterile gauze pad so that touch preparations of the slide can be made by the laboratory assistant, especially if an aspirate was not obtained as in the case of a "dry tap."
7. Upon completion of the biopsy portion of the procedure, pressure is applied to obtain adequate hemostasis; such will take longer in the setting of a patient who has a coagulopathy or thrombocytopenic state. The area is cleaned with a disinfectant and a sterile bandage; the latter can be removed after 24 h. Enhanced pressure and hemostasis can be accomplished by having the patient lie supine on a small towel rolled up into a flat ball using the person's own weight to apply the needed pressure.
8. The bone marrow aspiration and biopsy site should be inspected for delayed bleeding or infection over the next several days.

(d) *Post-procedure issues.* Patients and those who accompany the patient to the procedure should be instructed to watch for signs of bleeding at the biopsy site. If significant problems arise such as light-headedness, severe pain, altered mental status, or other adverse events, the patient should immediately seek medical attention.

8. *Collection of bone marrow for therapeutic intent:* Hematopoietic stem cells have been used for the restoration of lympho-hematopoietic function after myeloablative, reduced-intensity, and nonmyeloablative conditioning regimens. More recently, marrow cells are being used increasingly in clinical initiatives such as tissue repair and regenerative medicine.

A number of decades ago, the bone marrow was the only source of hematopoietic progenitor and stem cells. In the last two decades, there has been a shift to using marrow progenitor/stem cells mobilized into the peripheral blood via mobilizing stimuli such as filgrastim (G-CSF) and other agents. At present, only 20% of all graft sources are derived directly from the bone marrow. Blood mobilized progenitor/stem cells contain a tenfold higher content of T cells and other accessory cells compared to the bone marrow and therefore carry an increased risk of inducing graft-versus-host disease (GvHD). As a result, the bone marrow remains the preferred graft source for transplantation in selected non-neoplastic conditions such as severe aplastic anemia, sickle anemia, thalassemia, and genetic or acquired bone marrow failure syndromes (see Chap. 37 on "Hematopoietic Cell Transplantation") [18–20].

In general, the bone marrow harvest procedure is performed under spinal or general anesthesia in an operating room while the patient is in the ventral supine position. The procedure is essentially the same as that of the diagnostic marrow examination but is repeated many hundreds of times through six to eight posterior iliac crest skin site punctures. Total volume collected is usually based on the weight of the recipient but must be less than 20 mL/kg donor weight.

Below is the link to a marrow cell collection procedure video put forth by the National Marrow Donor Program. This video illustrates the current best practices in standard marrow collection methods. Physicians and health-care professionals performing this procedure should be educated in the optimal techniques in order to ensure safety of the donor while collecting the best possible product.

https://network.bethematchclinical.org/education/transplant-center/operational/transplant-center-staff-training/marrow-collection-procedures-video/.

Expertise in the bone marrow harvest procedure is critical to protect the donor while attaining a good cell dose for the recipient. The overall decline in the number of bone marrow harvests may translate into an overall lack of exposure of the procedure for bone marrow harvest team members [21–23]. Centers that perform fewer procedures also have more toxic events in the bone marrow donor [21]. Marrow harvesting by means of multiple small volume aspirations (approximately 1–3 mL) minimizes the dilution with peripheral blood and yields more nucleated cells and more colony-forming units (CFU-GM) than large volume aspirations [22].

C. *Morphologic examination of the bone marrow*

1. *Overview.* Procurement of bone marrow includes the bone marrow aspirate smears, bone marrow touch imprint (prep), bone marrow clot section, and bone marrow core biopsy (Fig. 21.5). Bone marrow aspirate smears and touch preps are performed primarily to evaluate the cytological features of marrow hematopoietic cells, whereas the bone marrow tissue core biopsy and clot sections are used to determine global changes such as marrow cellularity or infiltration of the marrow by mass lesions such as lymphoma or metastatic tumor. Smears are routinely stained with a Romanowsky stain such as Wright-Giemsa, whereas bone marrow biopsies are stained with hematoxylin and eosin similar to other surgical biopsy specimens.

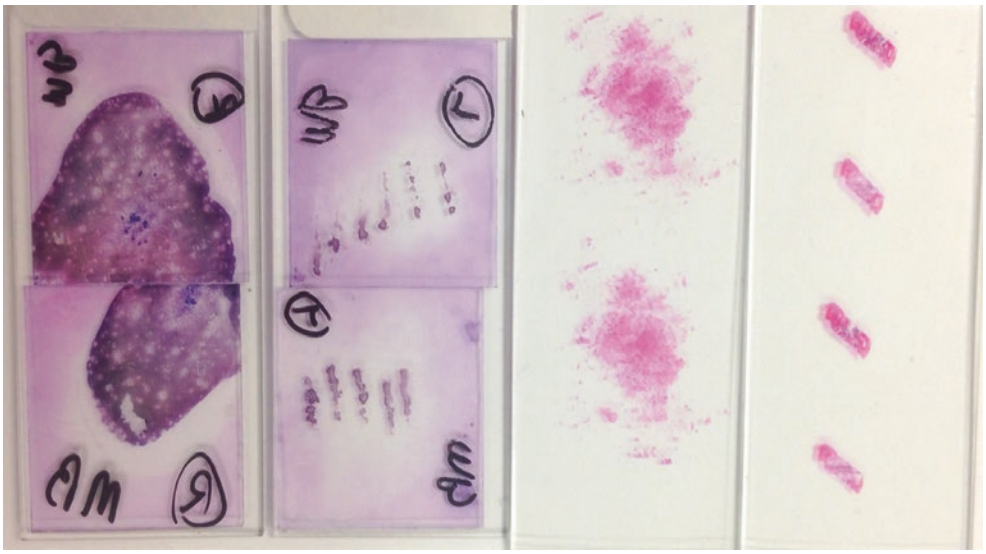

Fig. 21.5 Slide preparations of bone marrow aspirate smear, bone marrow touch prep, bone marrow aspirate clot, and bone marrow core biopsy

The Wright-Giemsa method stains acidic components of the cells blue or blue-purple and basic components light red or orange. The nucleus, composed predominantly of DNA, is therefore stained dark blue by this process, and proteins in the cytoplasm, in general, are basic and as a result stain light red/orange. Regions rich in carbohydrates do not react with either method and appear clear, including the Golgi apparatus and nucleoli. The hematoxylin and eosin stain is similar to the Wright-Giemsa although nucleoli are red-purple rather than clear. To further aid in the diagnosis and evaluation of hematopoietic disorders, ancillary studies are necessary such as histochemical and immunohistochemical stains, flow cytometry, and cytogenetics.

2. *Bone marrow aspirate*
   (a) Adequacy. Adequacy of the marrow aspirate smear can be determined by the presence of visible marrow particles or spicules (Fig. 21.6) [3]. The unfortunate use of the term spicule leaves the erroneous impression that small bone fragments are present on the aspirate smear. In fact, the particles are mini-pieces of bone marrow aspirated into the syringe maintained as small tissue fragments. Actual bony fragments are not present. A good quality aspirate smear should contain one or more marrow particles.
   (b) Low-power examination (40× or 100×):
      1. The bone marrow aspirate smear should first be viewed under low-power magnification to determine the number of particles. One also assesses the quality of the aspirate to determine the adequacy of the sample.
      2. Megakaryocytes, metastatic tumor cells, and other rare elements are best scanned for at low power since they are not abundant.
      3. Low-power examination is a necessary first step. Jumping to a high-power examination results in the morphologist overlooking items to examine.
   (c) High-power examination (400×, 500× or 1000×):
      1. Areas to review:
         (a) Areas of well-preserved cells near the edges of bone marrow particles should be identified at low power and used for high-power examination.
         (b) An oil immersion lens (500× or 1000×) should be used liberally.
         (c) Examining cells within particles is difficult due to overlapping cells and the presence of many broken cells. Additionally, the thickness of the smears in the spicules often prevents the Romanowsky stain from fully penetrating the cells leading to an artificially pale stain.
         (d) Examination of the smear far away from particles is also to be avoided since these areas are significantly diluted by peripheral blood.
      2. Bone marrow cells:
         (a) Granulocytes:
            (i) The granulocyte stages are on a continuum. As neutrophil maturation progresses, cell size becomes progressively smaller, granulation becomes more prominent, the nuclear/

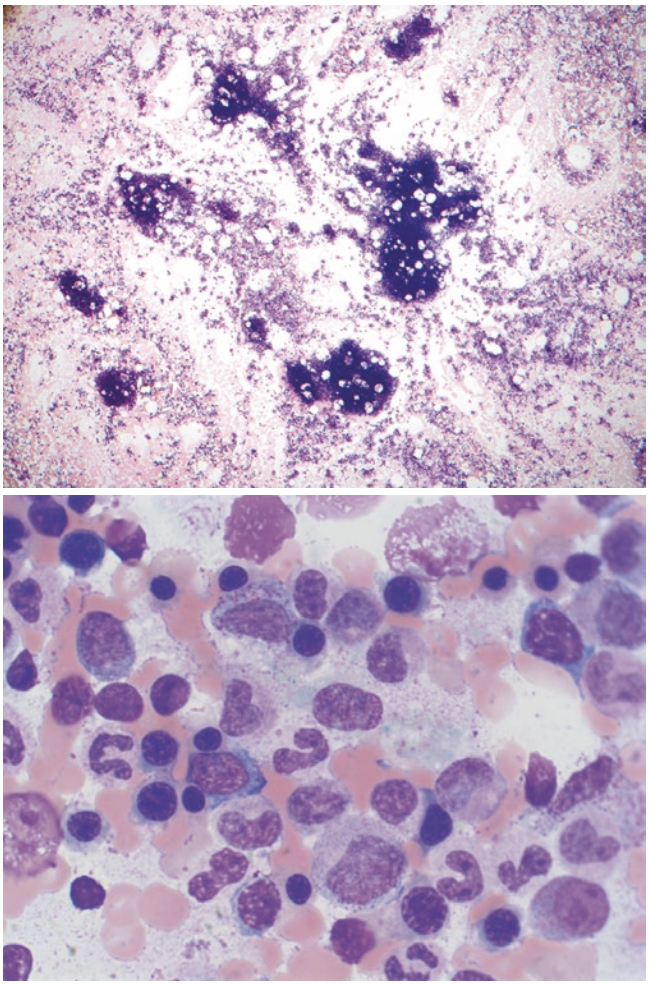

Fig. 21.6 Normal bone marrow aspirate, Wright-Giemsa stain. Low-power magnification (40×), top, showing spicules or bone marrow fragments, an indication of good marrow sampling. High-power examination (1000×), bottom, allows for the evaluation of the cytologic features of marrow hematopoietic elements, primarily granulocyte and erythroid precursors. Cell differential counts are routinely performed on bone marrow aspirate smears

cytoplasmic (N/C) ratio decreases, and the nucleus indents and finally segments. Although distinct phases are recognized, any one particular cell may not be able to be classified into a specific stage. Identifiable cell types include the promyelocyte, myelocyte, metamyelocyte, and band and segmented forms (Fig. 21.7).

(ii) *Promyelocyte*. This is the first recognizable cell in the granulocyte pathway. A promyelocyte is the largest granulocyte, 15–25 μm in size, and contains an oval nucleus with modestly condensed chromatin, a high nuclear/cytoplasmic (N/C) ratio, with the nucleus occupying roughly 70% of the cell area and a single distinct nucleolus. The cell only expresses the reddish-purple primary granules in the cytoplasm which are sparse and occasionally overly the nucleus (Fig. 21.7).

(iii) *Myelocyte*. The myelocyte stage heralds the first period where the specific granulocyte type (neutrophil, eosinophil, or basophil) can be recognized. Most granulocytes develop into neutrophils. As a result the vast majority of myelocytes and later forms are of neutrophil lineage. These cells are smaller than promyelocytes, 12–20 μm in size, and have an oval nucleus with moderately high N/C ratio with the nucleus occupying roughly 60% of the cell area and lacking a distinct nucleolus. The cytoplasm is notable for the presence of both reddish-purple primary granules and tan-colored neutrophil secondary granules. Additionally, the cells demonstrate a prominent Golgi apparatus recognized as a clear area adjacent to the nucleus (Fig. 21.7).

(iv) *Metamyelocyte*. The next stage in neutrophil development is the metamyelocyte. These cells are 12–15 μm in size with a further reduction in the N/C ratio to where the nucleus occupies roughly 40% of the cell area. The nuclear chromatin is more condensed (darker) than the previous stages and begins indenting. The cytoplasm is similar to the mature neutrophil with tan-colored secondary granules without visibly detectable primary granules (Fig. 21.7).

(v) *Band*. The band is similar to the metamyelocyte although slightly smaller, 10–15 μm, with a condensed dark-staining nucleus. The major distinction is the nucleus is indented more than 50% of its width whereas metamyelocytes nucleus indents to a lesser degree. Lack of nuclear segmentation, the presence of nuclear string between nuclear lobes, distinguishes the band form from the segmented neutrophil (Fig. 21.7).

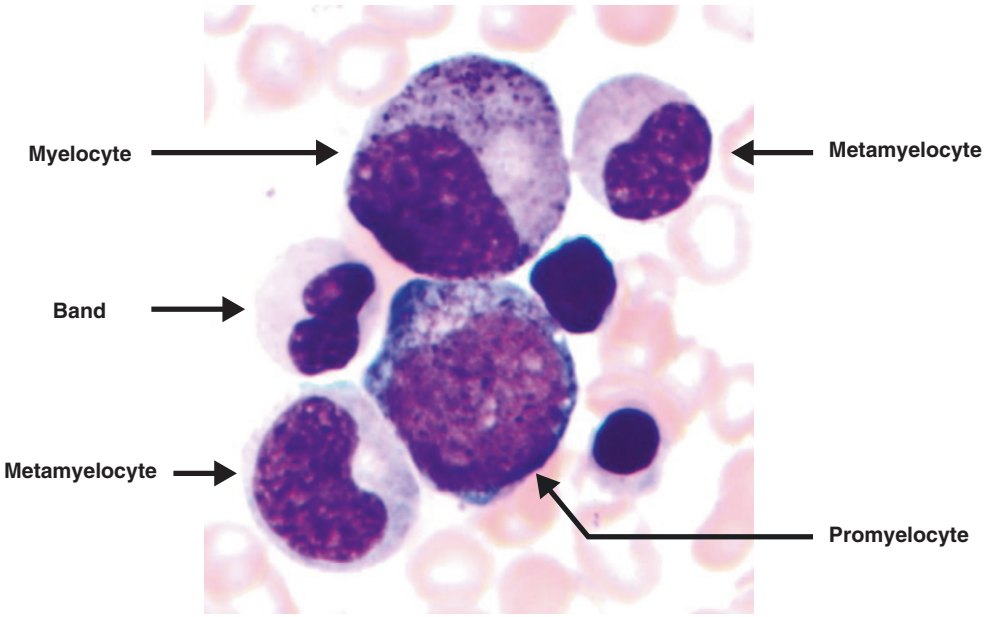

Fig. 21.7 Granulocytic cells on bone marrow aspirate smear (1000×). Stages are indicated

(vi) *Segmented form*. The segmented form is similar in size to the band but contains a nucleus with three to five distinct nuclear lobes or segments.

(b) Erythroid precursors. Red cell progenitors, like granulocytes, are on a developmental continuum. Red cell progenitors at all stages are notable for very round centrally placed nuclei. During maturation, the cells become progressively smaller, and the nucleus shrinks, condenses, and is ultimately extruded. Simultaneously the cytoplasm converts from a RNA-rich milieu to one composed predominantly of hemoglobin changing from dark blue to light red in color. Identifiable cell types include the proerythroblast, basophilic erythroblast, polychromatophilic erythroblast, and orthochromic erythroblast (Fig. 21.8).

(i) *Proerythroblast*. These cells are relatively large, 15–20 μm, with high N/C ratio with the nucleus occupying roughly 80–90% of the cell area. The nucleus has a finely stippled or granular structure with several small nucleoli. The cytoplasm is strikingly dark blue (basophilic) due to the high RNA content without granules (Fig. 21.8).

(ii) *Basophilic erythroblast*. This is the next phase of erythrocyte development. These cells are slightly smaller with a slightly reduced N/C ratio with the nucleus occupying approximately 70–80% of the cell area. The nucleus is more condensed, without nucleoli and has small open areas or cracks imbuing a checkerboard-like appearance to the cell. The cytoplasm remains intensely basophilic and without granules (Fig. 21.8).

(iii) *Polychromatophilic erythroblast*. As maturation progresses the red cell enters the polychromatophilic erythroblast stage. These cells are smaller than basophilic erythroblasts with further reduction in the N/C ratio with the nucleus down to roughly 60% of the cell area. The nucleus is condensed, dark staining, with small open areas and cracks remaining. The cytoplasm begins to show evidence of hemoglobin production as RNA content is reduced. This results in the cytoplasm displaying both reddish (hemoglobin) and blue (RNA) colors giving rise to the name polychromatophilic, or multicolored, cell (Fig. 21.8).

(iv) *Orthochromic erythroblast*. The final recognizable marrow phase of red cell development is the orthochromic erythroblast. This is the last stage in which the red cell retains its nucleus. The cell is small and approaches the size of a peripheral blood red cell. The nucleus is very condensed, pyk-

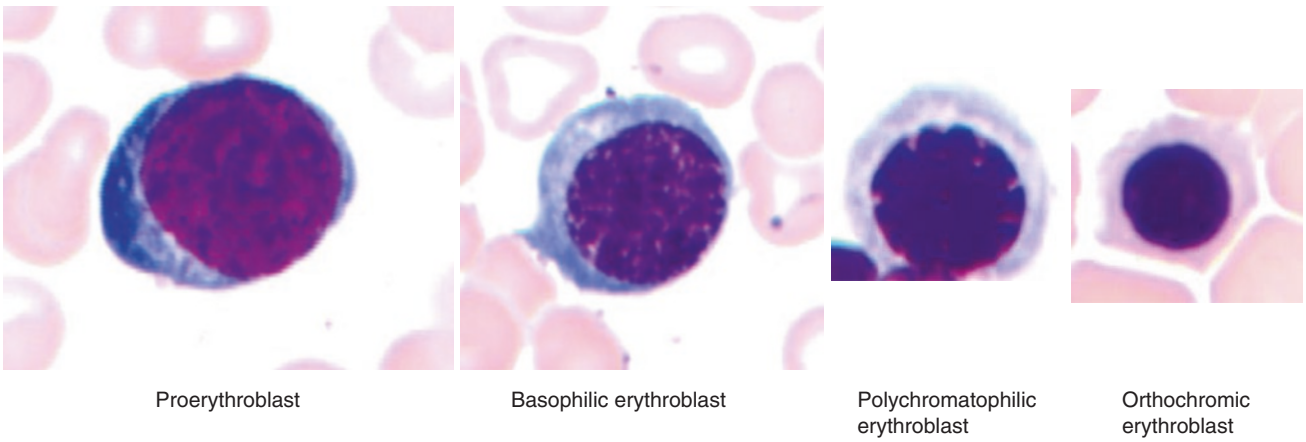

Fig. 21.8 Red cell progenitors, erythroblasts, on the bone marrow aspirate smear (1000×). Stages are indicated

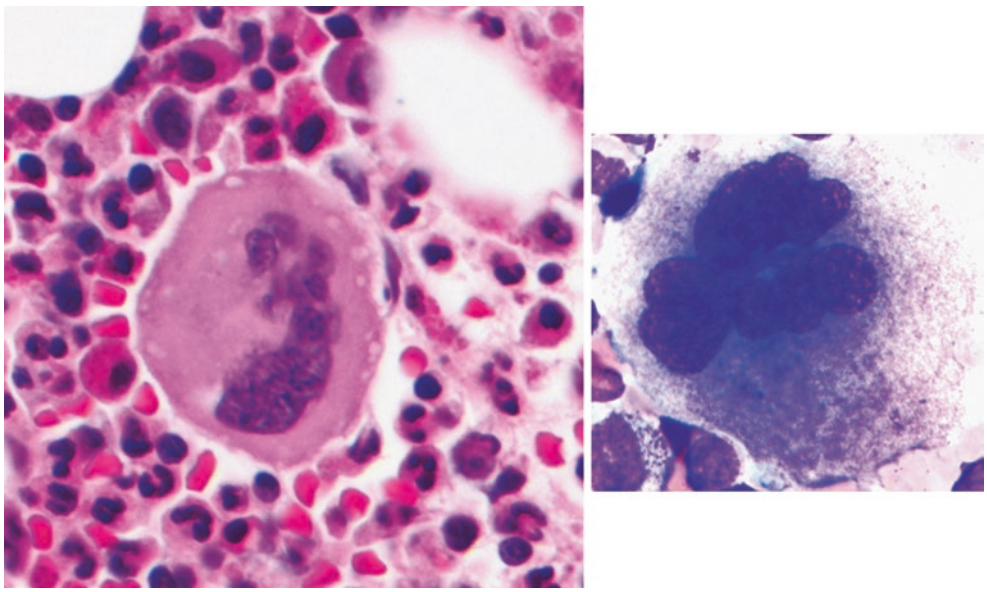

Fig. 21.9 Megakaryocyte on bone marrow core biopsy (1000×), left, and bone marrow aspirate smear (1000×), right

notic, and homogeneously dark staining. The N/C ratio is low with the nucleus down to roughly 40% of the cell area. The cytoplasm is further hemoglobinized (red) with only a subtle blue tinge indicative of low-level remaining RNA similar to the peripheral blood reticulocyte (Fig. 21.8).

(c) *Megakaryocyte*. Megakaryocytes are rare constituting roughly 1/1000–1/10,000 of other marrow progenitors although easily recognized due to their very large size (35–160 μm). The cells are unusual as they undergo nuclear duplication without cytoplasmic division, a process referred to as *endomitosis*. A typical megakaryocyte may undergo five or six rounds of nuclear division giving rise to DNA content of 32–64 N compared to the normal 2 N of a typical hematopoietic precursor. This process results in the cell's large size and a lobulated nucleus with overlapping nuclear lobes. The cytoplasm is abundant resulting a relatively low N/C ratio and morphology similar to that of a mature platelet containing many small azurophilic (purple or blue-red) granules (Fig. 21.9).

(d) *Blast*. Most blasts in the marrow are destined to differentiate toward non-lymphoid (myeloid) cells. Morphologically distinguishing between blasts committed to different lineages is not possible without special markers. These cells are the most primitive cell recognized in the marrow and are present at low levels usually 1% or

less. Blasts are medium-sized cells, 15–20 μm, with a high N/C ratio occupying roughly 80% of the cell area. The nucleus is oval to round with finely dispersed chromatin making the nucleus significantly paler than that of a lymphocyte. Occasionally small nucleoli may be present. The cytoplasm is blue-grey and lacks granules (Fig. 21.10).

(e) Others:

(i) *Lymphocytes*. Lymphocytes in the marrow are morphologically similar to those in the peripheral blood. In young children and after cytotoxic therapy, immature lymphocytes are encountered referred to as *hematogones* (Fig. 21.10). These cells are similar to normal mature lymphocytes but are larger with very high N/C ratios.

(ii) *Plasma cells*. Plasma cells represent the end-stage of B cell development. A low percentage of these cells are encountered in a normal bone marrow aspirate. The cells are slightly larger than a lymphocyte. The nucleus is round to oval, condensed, and eccentrically placed and occupies a small area of the cell (low N/C ratio). The nucleus often demonstrates a pattern of open cracks that resembles a clock face. The cytoplasm is dark blue with a large perinuclear Golgi apparatus giving a clear "halo" adjacent to the nucleus. This is a characteristic feature of the cell. Plasma cells are often confused with basophilic erythroblasts. The distinction can be made by noting the eccentric nucleus and prominent Golgi apparatus of the plasma cell lacking in the basophilic erythroblast (Fig. 21.11).

(iii) *Monocytes*. Monocytes are not easily identified on the aspirate smear and are often confused with myelocytes. As opposed to the myelocyte, the cell has a U-shaped or indented nucleus, and although monocytes contain scattered small red-purple granules similar to primary granules, they lack secondary granules. The cell cyto-

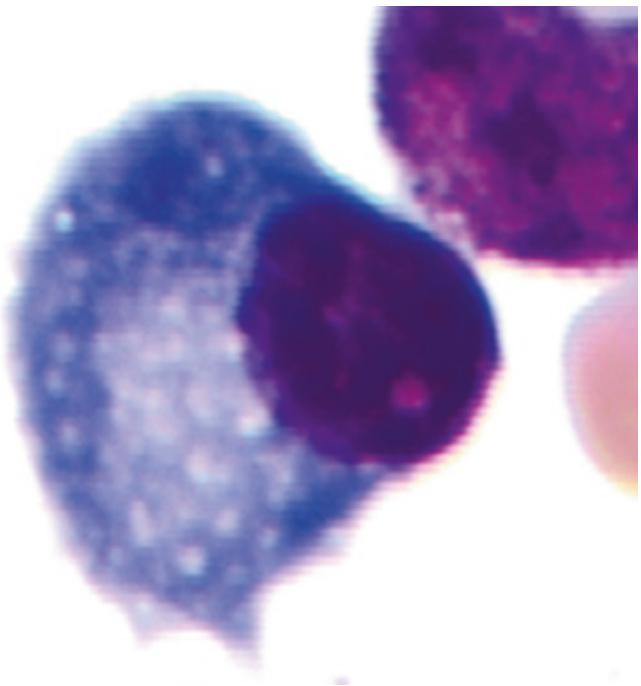

Fig. 21.11 Plasma cell on bone marrow aspirate smear (1000×)

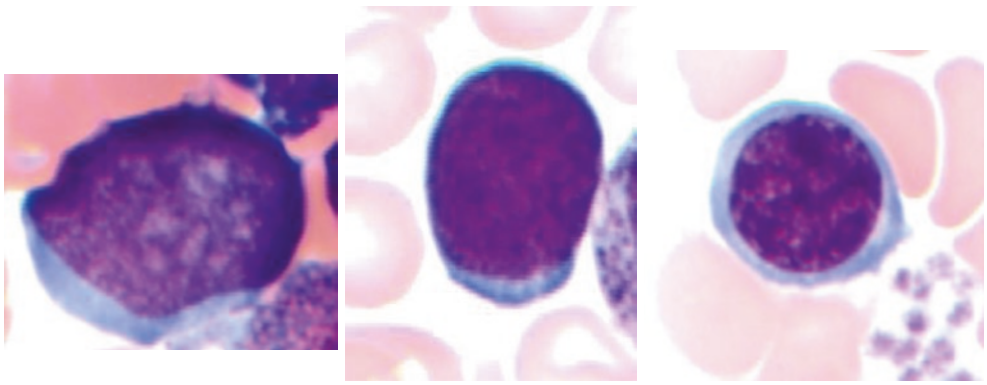

Fig. 21.10 Bone marrow aspirate smear (1000×) showing myeloid blast (left) B cell progenitor also referred to as B lymphoblast or hematogone (center) and mature lymphocyte (right)

plasm is also notably grey to light blue in color. Immature monocyte stages, *promonocytes*, are also present in the marrow but are generally not recognizable.

(iv) *Macrophages*. Macrophages represent the tissue phase of a monocyte and are scattered throughout the marrow aspirate smear, particularly at the edges and in and around marrow particles. The cells are large with very low n/c ratios with abundant pale blue to clear bubbly cytoplasm not uncommonly containing cell debris. The nucleus is oval to triangular-shaped or sometimes flattened with modestly condensed chromatin and often at the edge of the cell. Macrophages are more noticeable in bone marrow specimens whenever the overall cellularity is low such as after chemotherapy or bone marrow injury (Fig. 21.12).

(v) *Mast cells*. Mast cells are similar to but not direct descendants of basophils. They have different progenitor cells. They are encountered occasionally in the bone marrow aspirate particularly when lymphoid aggregates are present. Mast cells are usually seen within the marrow particles intimately associated with the marrow stroma. The cells are easily identified based on their prominent purple-blue cytoplasmic granules which partially obscure the nucleus. The granules are relatively large and refractive. The cells often fall apart upon making of the smear resulting in cells with granules spilling out of the cytoplasm (Fig. 21.13).

(vi) Non-hematopoietic elements:

(a) Osteoblasts and osteoclasts were described above (Figs. 21.2 and 21.3).

(b) Stromal cells were also described above. Stromal cells are the main constituents of the particles although often obscured by hematopoietic elements. In hypocellular specimens stromal cells are seen as groups of cells with elongated nuclei and spindle-shaped feathery cytoplasm.

3. Bone marrow differential:

(a) A minimum of 200 cells are generally counted and classified to determine a bone marrow differential count (Table 21.2).

(b) All neutrophil granulocyte precursor stages are enumerated (promyelocyte, myelocyte, metamyelocyte, band, segmented neutrophils) as well as eosinophils, basophils, erythroid progenitors, monocytes, lymphocytes, and plasma cells.

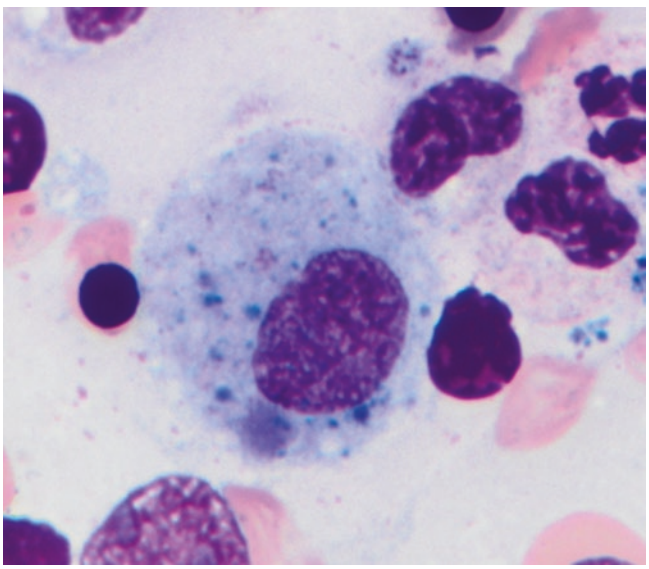

Fig. 21.12 Macrophage on bone marrow aspirate smear (1000×)

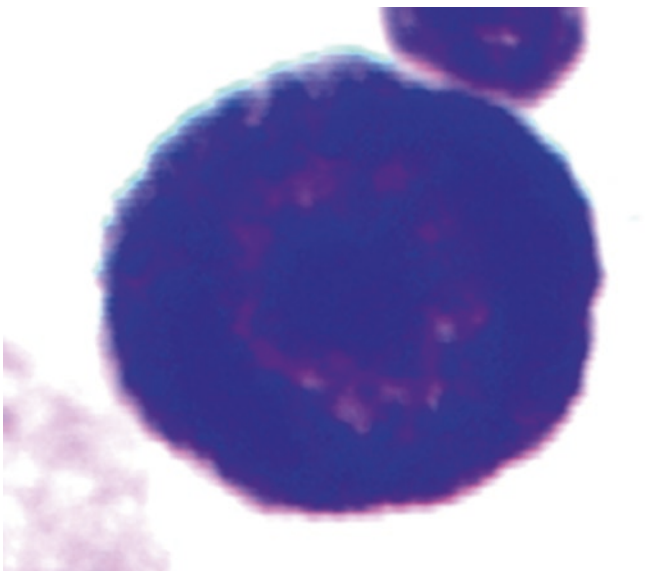

Fig. 21.13 Mast cell on bone marrow aspirate smear (1000×)

Table 21.2 Normal bone marrow differential count

	Normal range (%)
Promyelocyte	1–5
Myelocyte	5–10
Metamyelocyte	10–25
Band	10–20
Segmented neutrophil	5–30
Eosinophil	2–4
Basophil	0–1
Blast	0–1
Lymphocyte	5–25
Monocyte	0–2
Plasma cell	0–2
Erythroid cell	17–35
Myeloid/erythroid	1.5–3

(c) The various stages of erythroid maturation are not usually specifically distinguished in the marrow differential.

4. Myeloid/erythroid ratio (M/E ratio):
   (a) The cell count includes only hematopoietic elements and is used to determine the *myeloid/erythroid ratio* (M/E ratio).
   (b) The normal M/E ratio is the ratio of the number of all granulocytic elements to the number of erythroid cells.
   (c) It is normally 1.5–3 to 1 and remains fairly steady throughout life. Only the relative distribution of granulocytes to erythroid cells can be determined on aspirate smears.
   (d) The M/E ratio can be affected by the quality of the smear and areas of the smear examined.
      (i) A bone marrow smear devoid of particles and diluted by peripheral blood generates a high M/E ratio with many mature segmented neutrophils due to the inclusion of peripheral blood elements.
      (ii) Enumerating cells away from marrow particles will also lead to an overestimation of the M/E ratio due to inclusion of peripheral blood cells in these areas.

(d) Cell distribution:
1. Cellular elements do not distribute evenly on the aspirate smear.
2. Larger cells, primarily granulocyte precursors, tend to be dragged to the edges.
3. Plasma cells, mast cells, and megakaryocytes often remain close to the marrow particles.
4. Lymphoid collections may be found focally on aspirate smears due to aspiration of a lymphoid aggregate.
5. All areas of the smear should be examined to generate an estimate of the relative distribution of cell types, and the zones chosen for cell counts should sample the most representative areas.

(e) Adequacy of megakaryocytes:
1. Counting megakaryocytes on aspirate smears is not considered reliable.
2. Megakaryocytes tend to reside in or near marrow particles and can get caught up in small clots during the aspiration procedure making them difficult to detect.
3. A general sense of the relative abundance of megakaryocytes is assessed in a good quality, un-clotted aspirate to at least determine whether a thrombocytopenia is the result of peripheral destruction (many megakaryocytes) or marrow failure (few megakaryocytes). The best method to assess the adequacy of megakaryocytes, however, is the bone marrow core biopsy.

3. *Bone marrow core biopsy*
   (a) Adequacy:
   1. Bone marrow core biopsies should be 1.5 cm in length and contain at least five intertrabecular spaces (Figs. 21.14 and 21.15) [4, 5].
   2. The likelihood of detecting metastatic tumor or marrow involvement by lymphoma is dependent on the length of the marrow biopsy. Marrow biopsies shorter than 1.5 cm are less sensitive for the detection of solid tumors. Minimal additional sensitivity is gained above this length [4, 5]. In children, biopsies are shorter by necessity requiring bilateral cores for the evaluation of metastatic disease.
   3. It is debatable whether bilateral biopsies add significantly to an otherwise adequate 1.5 cm unilateral biopsy in adults for the staging of lymphoma or detection of metastatic tumor.

   (b) Processing:
   1. Biopsy specimens are handled much like other tissue biopsies although the hardness of the bone requires removing calcium with a weak acid during the fixation process to allow microtome sectioning.
   2. The specimen is fixed in formalin or similar fixative, processed, and embedded in paraffin blocks. Several shavings of the block are taken and laid onto glass slides whereupon they are stained with hematoxylin and eosin (H&E).
   3. Additional stains such as a reticulin are performed for specific indications. Iron stains are not reliable on core biopsies since iron may leech out during the decalcification process.

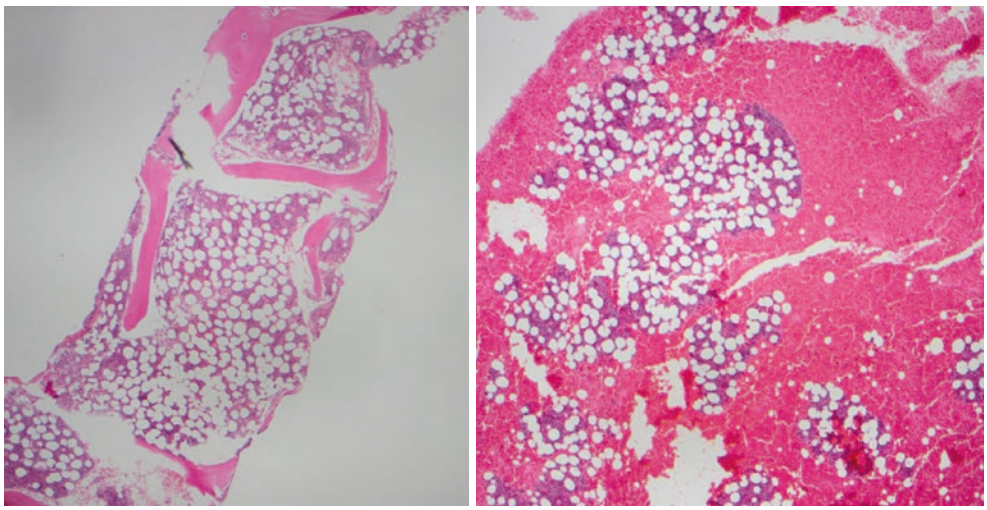

Fig. 21.14 Normal bone marrow biopsy, right, and bone marrow aspirate clot, left, hematoxylin and eosin stain (40×). Low-power magnification helps to assess the cellularity of the bone marrow biopsy and adequacy of megakaryocytes and evaluate for infiltrative processes

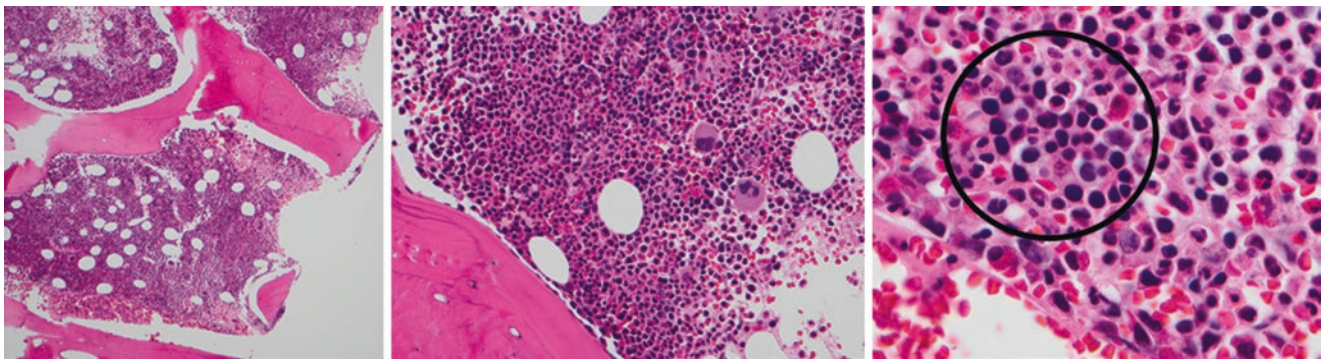

Fig. 21.15 Normal bone marrow biopsy hematoxylin and eosin stain. 100×, left; 200×, center; 500×, right. Medium- and high-power examination allows for determination of megakaryocyte morphology and cytologic assessment of other marrow elements and infiltrative processes, if present. A group of red cells progenitors can be identified on the 500× image (circle)

(c) Low-power examination (40×, 100×):
   1. As for bone marrow aspirate smears, bone marrow biopsy specimens should first be scanned at low power prior to examining at higher-power magnification.
   2. Evaluation of biopsy sections with an oil immersion lens is not necessary.
   3. A general scan at low power is helpful to determine the adequacy of the specimen, assess marrow cellularity, and identify focal processes such as granulomas, lymphoid aggregates, and metastatic tumor.
   4. Adequacy of megakaryocyte numbers is best determined on the core biopsy at low-power magnification.

(d) High-power examination (200×, 400×):
   1. Higher-power magnification is useful to assess the distribution and nature of the hematopoietic cellular elements and bone structure and to evaluate the details of infiltrative processes, if any.

(e) Assessment of cells and cellular structures:
   1. A judgment of the increase or decrease of the granulocyte or erythroid cells can be made from the core biopsy in conjunction with the aspirate smear cell counts. A hypocellular core biopsy with a decreased M/E ratio indicates a granulocytic hypoplasia, whereas a hypercellular core biopsy with a low M/E ratio reflects an erythroid hyperplasia.
   2. Bony trabeculae should be evaluated for evidence of increased bony remodeling and trabecular thickness. Increase in bony remodeling may indicate hyperparathyroidism due to renal disease, a previous injury or biopsy, or a solid malignancy inducing reactive bony changes. Lymphomas have a predilection for growing adjacent to the bony trabeculae, especially follicular lymphoma.

3. Marrow sinuses should be collapsed. Dilated or drawn open sinusoids suggest a pathologic increase in reticulin fibrosis.
4. Hematopoietic cells in the normal bone marrow will demonstrate some zonation with erythroid progenitors and granulocyte progenitors remaining in loose ill-defined collections. This zonation is enhanced in the recovery phase after chemotherapy or hematopoietic stem cell transplant. Cells should be arranged in large cords. "Lining up" of the hematopoietic cells in parallel streams is an indication of marrow reticulin fibrosis.
5. All stages of hematopoiesis should be evident although this is best judged on the aspirate smears.
   (a) Erythroid progenitors contain very dark "ink dot" nuclei that are round. Erythroid cytoplasm has distinct cell borders under high-power magnification.
   (b) Granulocytes progenitor nuclei are irregular to oval, depending on the stage of development, and are pale. Cytoplasm is granular, slightly refractive, and pink to red.
   (c) Eosinophil cytoplasm is strikingly orange to orange-red with strongly refractive granules. The number of eosinophils is easy to overestimate on a core biopsy since their color attracts the morphologists' eye.
6. Megakaryocytes:
   (a) Megakaryocytes are generally scattered, peri-sinusoidal, and easily recognized based on their increased size compared to other hematopoietic cells (Fig. 21.9).
   (b) Between 7 and 15 megakaryocytes per mm^2 should be present on marrow sections [4]. However, adequacy is most often assessed subjectively. Megakaryocyte numbers should mirror overall cellularity.
   (c) Large, focal collections of megakaryocytes are pathologic.
   (d) Megakaryocyte nuclei should have multiple overlapping nuclear lobes. However, not all lobes of the megakaryocyte are visible in the plane of sectioning on an H and E section. Therefore, assessing megakaryocytes for small hypo-lobulated nuclei can be difficult.

4. *Bone marrow aspirate clot*
   (a) Overview. Aspirated marrow left in the aspirating syringe not used to generate smears is an additional source of material that can be used for morphologic examination. This aspirated material is left to clot (*aspirate clot*) and can be fixed and sectioned similar to the core biopsy allowing review of supplementary marrow tissue fragments (particles) caught up in the clot (Figs. 21.5 and 21.14).
   (b) Use. The morphologic features of the clot are similar to that of the core biopsy, but clot sections lack bony fragments. Most laboratories will use clot sections to maximize morphologic examination of the bone marrow. Additionally, Perl's Prussian blue stain for iron stores can be performed on the clot sections since hemosiderin is not lost during processing.
5. *Bone marrow touch imprint*
   (a) Overview. Occasionally bone marrow cannot be aspirated from a patient (see above) due to hypocellularity, fibrosis or extensive infiltration of the marrow by a neoplastic process. This is referred to as a "*dry tap*." To view the individual cellular elements of the marrow, a *touch imprint* or *touch prep* can be performed (Fig. 21.5).
   (b) Method. A fresh core biopsy specimen is physically touched to a slide depositing cells from the marrow on the glass. Multiple light imprints should be performed as the first touches will contain adsorbed peripheral blood elements. Touch imprints, if performed correctly, give the most representative distribution of marrow cellular elements.
   (c) The evaluation of touch preps is identical to that of the aspirate smear.
   (d) Artifacts. The pressure of imprinting cells on a slide can distort cell morphology leading to artifacts. The force of touching a core biopsy to glass leads to spreading of a cell over a slightly larger area on the slide compared to that on the aspirate smear. As a result cells and cell structures on a touch imprint appear paler and larger than on an aspirate. A benign lymphocyte, appearing larger with a pale nucleus, can easily be misinterpreted for a blast in such circumstances.
6. *Iron stain*
   (a) Overview. Due to the importance of iron for erythropoiesis, stains for iron stores are routinely performed on bone marrow specimens. Microscopic examination of bone marrow stained for iron is considered the gold standard for determining iron-depleted states. Iron stains can be done on the bone marrow aspirate smear or a bone marrow clot section or both.

(b) Method. *Perl's Prussian blue stain* is the method of choice for iron staining. The stain generates a blue color when *hemosiderin* is present. Sections are often counter-stained with safranin, a red dye, to enhance the visualization of the stained iron (Fig. 21.16).

(c) Evaluation:

1. Iron stores are evaluated by examining several particles on the aspirate smear or clot section. A marrow specimen lacking particles is insufficient to determine iron stores.
2. Marrow iron is present within macrophages and erythroid progenitors.
   (a) Erythroid cells containing iron are referred to as *sideroblasts*. Iron in sideroblasts is present as one or two fine siderotic granules best visualized with an oil immersion lens (1000×).
   (b) Siderotic granules in macrophages should be small, few to moderate in number, and visible under low-power magnification (100×).
3. Stainable iron is in the form of *hemosiderin*, a macromolecule complex of *ferritin* and iron.
4. Iron stores are assessed subjectively, as a practical matter, as absent, reduced, normal, or increased. Iron visible only in a few cells under high-power magnification (1000×) is decreased, whereas iron in many cells aggregating in clumps is an indication of iron overload.
5. *Ring sideroblasts* are defined as erythroid cells that contain five or more siderotic granules encircling one-third or more of the nucleus. These cells are seen only in pathologic states and are an indication of abnormal iron accumulation in the mitochondria.

(d) Clinical relevance:

1. Stainable iron is increased post-transfusion, in thalassemia, hemochromatosis, and in sites of previous hemorrhage.
2. In anemia of chronic disease, macrophage iron is increased and associated with decreased iron in sideroblasts.
3. Decreased stainable iron is present in iron deficiency and is normally difficult to identify in growing children.

7. *Ancillary tests*

(a) *Histochemical stains*

1. Overview. Stains that use a chemical to impart color to subset of cells, identify organisms, or highlight specific tissue or cellular components are referred to as *histochemical stains*. Perl's Prussian blue stain is one type of histochemical stain. Histochemical stains can be performed on the bone marrow aspirate smear or core biopsy.
2. *Reticulin*
   Reticulin is visualized using a silver impregnation technique. The stain is only used on tissue sections. Reticulin should be assessed in marrow biopsies in which there was a "dry tap," in disease states known to be accompanied by increased reticulin fibrosis and where the H and E sections suggest increased reticulin formation (Fig. 21.17).
3. *Special stains for acute leukemia*
   These stains include myeloperoxidase, sudan black B, butyl esterase (with and without fluoride), chloracetate esterase (leder), and periodic acid Schiff (PAS). The stains are used on aspirate smears mostly to highlight the differentiation state of blasts. They are primarily of historical interest since most have been replaced by more specific antibody stains and flow cytometry.
4. *Stains for microorganisms*
   These stains are used on tissue biopsy sections and should be considered when there is a granuloma or other evidence to suggest an infection. The most commonly used stains are *Grocott's methenamine silver (GMS)* stain to highlight yeast and fungi and the *Ziehl-Neelsen (AFB)* stain to locate mycobacteria.

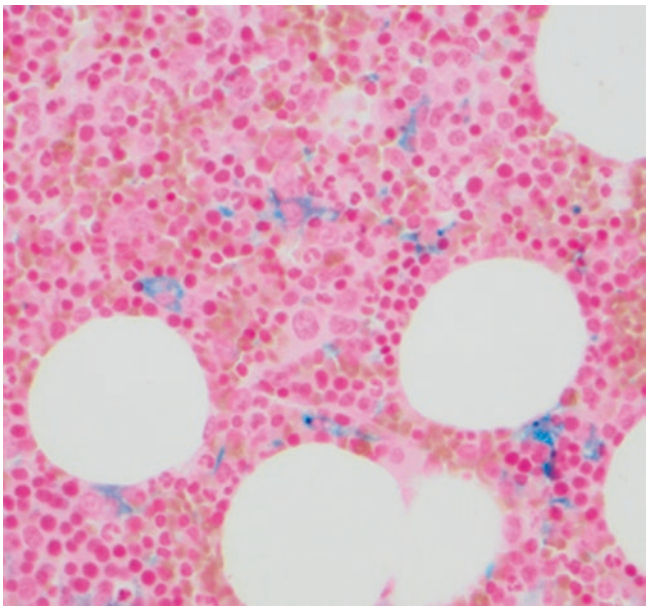

Fig. 21.16 Bone marrow Prussian blue stain for iron. Iron stains a light blue color and is localized primarily in the marrow macrophages. Bone marrow clot section (200×)

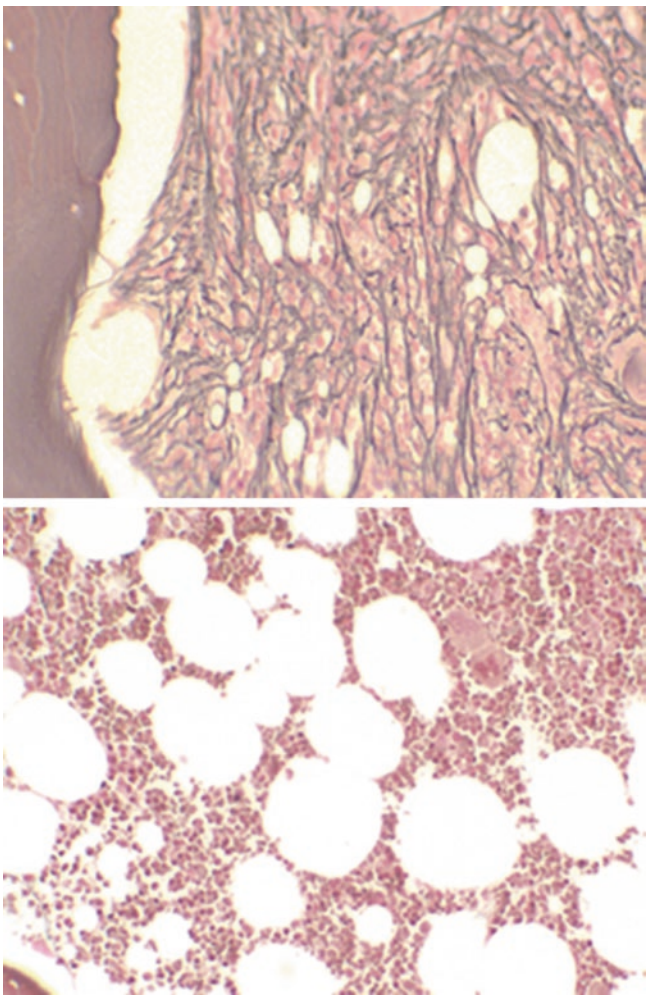

Fig. 21.17 Reticulin staining of bone marrow. Normal bone marrow reticulin stain, bottom. Only a few fine wisps of fine reticulin fibers are seen. Abnormal reticulin deposition, top. Course reticulin fibers are present encircling nearly all hematopoietic cells

5. *Stains for amyloid*
   Amyloid is an extracellular deposition of proteins in tissue into an insoluble beta-pleated sheet structure. It has a waxy, homogeneous, and eosinophilic appearance on an H and E stain. Amyloid may accompany plasma cell diseases. The *Congo red stain* is most often used to identify amyloid inducing an apple green color under polarized light.
6. Others
   Additional stains such as toluidine blue and giemsa for mast cells and trichrome for collagen deposition are used when there is a specific indication.

(b) *Immunohistochemical stains*

1. Overview. *Immunohistochemical stains* use antibodies to direct a chromogen (colored compound) to specific cells. This is carried out by adding an antibody of known specificity to a cut section of tissue on a glass slide. The site of antibody deposition is made visible through conjugation of the antibody to an enzyme, most often horseradish peroxidase or alkaline phosphatase, which can convert a chemical into a colored substance. Evaluation of the bone marrow is enhanced through the judicious use of immunohistochemical stains, a full discussion of which is beyond the scope of this chapter (see Chap. 28). Immunohistochemical stains allow for an assessment of the phenotype of cells while maintaining cellular morphology. The stains are used to determine the location and nature of specific cell subsets in the bone marrow. Immunohistochemistry is complimentary to flow cytometry for assessing cell antigen expression. Table 21.3 lists commonly used antibodies and cells they detect.
2. *Use*
   (a) Lymphoid aggregates. One of the more common uses of antibody stains is to determine the cellular composition of lymphoid aggregates in the bone marrow. Focal collections of lymphocytes in the bone marrow are common, and deciphering the nature of such aggregates (reactive versus neoplastic) requires phenotypic analysis including antibody stains. The relative distribution of T and B lymphoid cells within the aggregate can be examined using B and T cell-specific antibodies.
   (b) Plasma cells. Immunohistochemical stains are also useful to evaluate for clonality of plasma cells by demonstrating a skewed ratio of kappa expressing to lambda expressing plasma cells.
   (c) Other. Antibody stains are utilized to evaluate metastatic foci, examine for residual leukemia or lymphoma cells, and to bring out cellular elements that may be difficult to perceive on H&E-stained slides (mast cells, small megakaryocytes, blasts, etc.). Antibody stains should be used when the result will change the diagnostic interpretation or medical management.

(c) *Additional tests*

1. Flow cytometry. *Flow cytometry* is a method of cell phenotypic analysis almost always performed in conjunction with the bone marrow morphologic examination. An aliquot of the

Table 21.3 Typical markers used to assess bone marrow cells

Antigen	Method	Detects	Use
CD3	FC or IHC	T cells	Assess lymphoid aggregates and evaluate for T cell infiltrates
CD7	FC	T cells	Lineage of acute leukemia, assess lymphoid aggregates, evaluate for T cell infiltrates
CD10	FC or IHC	Precursor B cells, germinal center B cells	Lineage of blasts and B cell lymphomas
CD13	FC	Granulocytes and monocytes	Identify granulocytes and monocytes, lineage assignment of acute leukemia
CD14	FC	Monocytes	Identify monocytes, lineage assignment of acute leukemia
CD19	FC	B cells	Lineage of acute leukemia, assess lymphoid aggregates, and evaluate for B cell infiltrates
CD20	FC or IHC	B cells	Assess lymphoid aggregates, and evaluate for B cell infiltrates
CD33	FC	Granulocytes and monocytes	Identify granulocytes and monocytes, lineage assignment of acute leukemia
CD34	FC or IHC	Stem cells/blasts	Assess for blasts
CD45	FC or IHC	Hematopoietic cells except erythroid cells and platelets	Identify hematopoietic cells
CD41/CD61	FC or IHC	Platelets/megakaryocytes	Identify megakaryocytes, lineage assignment of acute leukemia
CD117	FC or IHC	Myeloid blasts, mast cells	Lineage assignment of acute leukemia, identify mast cells
CD138	FC or IHC	Plasma cells	Identify plasma cells
Kappa or lambda	FC or IHC	B cells/plasma cells	Assess for clonality
Myeloperoxidase	FC or IHC	Granulocytes, myeloid blasts	Lineage assessment of blasts and hematopoietic cells
TDT	FC or IHC	Lymphoid blasts	Lineage assessment of blasts
Glycophorin A	FC or IHC	Red cells/red cell progenitors	Identify red cell progenitors, lineage assignment of acute leukemia

FC flow cytometry, *IHC* immunohistochemistry

liquid bone marrow aspirate is used for the analysis. The technique will be discussed in Chap. 21.

2. Cytogenetic and molecular testing. Several tools are used to assess whether genetic abnormalities have been acquired in the hematopoietic cells, a hallmark of neoplasia. These include *cytogenetics*, the morphologic study of the structure and number of chromosomes in cells in disease states, and DNA-based methods collectively referred to as *molecular testing*. These are also performed on an aliquot of aspirated liquid marrow or, in the case of molecular testing, can be evaluated on fixed tissue. The methods will be detailed in Chaps. 22 and 23.

8. *Summary*
   (a) Testing involves a combination of morphologic examination and phenotypic and genetic assessment of the marrow. It has several components.
      1. Slides, in the form of smears, of liquid bone marrow and peripheral blood are made and reviewed.
      2. Slides of tissue sections from clotted bone marrow aspirate and the cylindrical core biopsy of bone are made and evaluated.
      3. An aliquot of anticoagulated liquid marrow (in EDTA or heparin) is evaluated using flow cytometry.
      4. A separate aliquot of anticoagulated liquid marrow (in heparin) is used for cytogenetic studies with an additional aliquot (in EDTA) used for molecular testing, if needed.
      5. Histochemical and immunohistochemical stains are performed on tissue sections as necessary.
   (b) Evaluation and interpretation of the bone marrow specimen must always be made in the context of the clinical situation.

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Flow Cytometry in Hematology

22

Howard Meyerson

Flow Cytometry

A. Overview. Flow cytometry is a technique of cell analysis that has become an integral part of diagnostic hematology and immunology. A *flow cytometer* is an instrument that detects fluorescence emitted from cells as they are excited by a laser in a flow stream, Fig. 22.1. *Fluorescence* is an innate property of a compound in which it absorbs light energy at one wavelength and emits energy at a second, longer, light wavelength. The detection methods available for emitted fluorescence are highly sensitive making fluorescence probes, in general, a valuable tool in biology. Modern flow cytometers are believed capable of detecting down to 100 molecules per cell. The instrument operates much like a typical hematology analyzer except that samples are manipulated prior to analysis by the addition of one or more fluorescent compounds of known specificity. Typically monoclonal antibodies chemically conjugated with fluorescent molecules are used. Cells expressing a particular antigen are tagged by the fluorescent antigen-specific reagent and then analyzed. Hydrodynamic focusing ensures that only one cell traverses the path of the laser light at a time allowing for linkage of the fluorescent signal with a particular cell. The fluorescence emitted is recorded as fluorescent intensity per cell with the degree of fluorescence roughly correlated with the amount of antigen expressed. Cells need to be dispersed, un-clumped, and viable when analyzed. Therefore, specimens must be procured in an anticoagulant (EDTA or heparin), received fresh without fixatives, and delivered in a timely fashion to the laboratory. These requirements are easily accomplished with bone marrow and peripheral blood samples.

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B. Flow Cytometer: Technical Aspects
   (a) Specimen processing
      (i) Any specimen in which individual viable cells are present such as blood, bone marrow, body fluids (cerebrospinal fluid, pleural fluid, peritoneal fluid), or fresh tissue (lymph node or spleen) can be used. Blood and bone marrow specimens must be procured in an anticoagulant to prevent clotting. For tissues, cells need to be dispersed by mechanical dissociation prior to staining and analysis.
      (ii) In general, fluorescent-conjugated antibody or a fluorescent compound of known specificity is added directly to an aliquot of sample referred to as *staining*. Instances in which there may be interfering soluble material, such as immunoglobulins, washing of the sample is required to remove the soluble substance prior to adding the antibodies. Antibodies are incubated for a short time (10–15 min) to allow binding to their antigens. For blood or marrow samples, non-nucleated red cells are removed by using a reagent that is known to lyse red cells (ammonium chloride) but preserve white blood cells. Nucleated red cells are unaffected by lysis. The number of red cells (300–500-fold more cells than white cells in peripheral blood) requires their removal to prevent interference with the overall analysis. When red cells need to be analyzed, lysis is not performed.
      (iii) Multiple aliquots of sample are generally used. In clinical practice, the adequate evaluation of a sample requires a large number (usually 20 or more) of antibodies to be added and examined for their reactivity on the cells. Since flow cytometers generally cannot detect this number of antigens simultaneously (see below), this requires staining several different aliquots of a specimen with antibodies.

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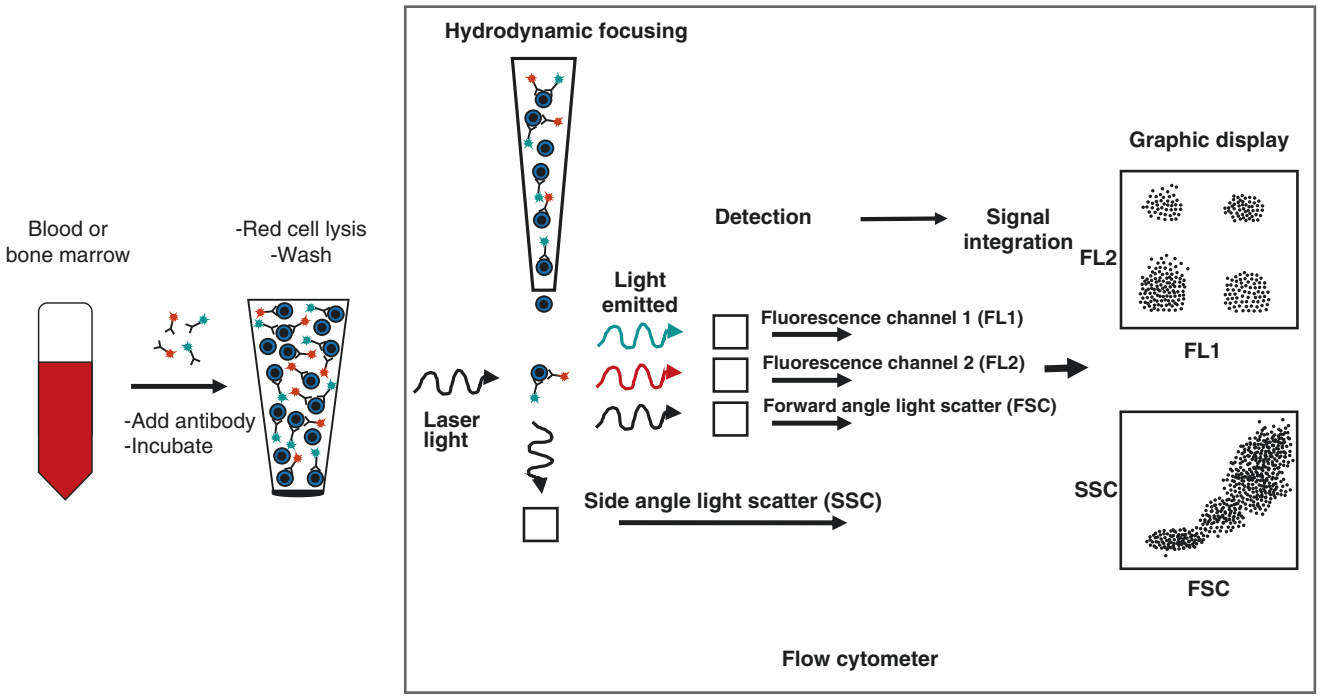

Fig. 22.1 Flow cytometry. Schematic of sample preparation and technical operations of a flow cytometer

1. Staining is generally performed in tubes. As such, separately stained aliquots are often designated as a stained "tube."
2. The aliquots needed are small. Peripheral blood contains 4–10 × 10^3 white blood cells per microliter. Therefore, a 100 µl aliquot enables the evaluation of hundreds of thousands of cells, if needed.

(iv) Surface vs intracellular staining. Most staining for flow cytometry is directed toward antigens expressed at the cell surface. Nonetheless, molecules present inside the cell including nuclear antigens also are assessed. Intracellular staining requires two steps in order for antibodies to access the interior of the cell without loss of cell integrity, a fixation step and a permeabilization step.

1. The lipid bilayer of the cell membrane functions as a barrier to the cell interior. In order for an antibody to penetrate the cell interior, bind, and stain an antigen, the lipid layer of the cell membrane must be broken down or removed. This is accomplished by the addition of a detergent. However, loss of cell membrane alone without first cementing the structure of the cell leads to cell dissolution and loss of the cellular unit. Hence, a fixation process to ensure rigidity of the cell is performed to maintain its structure prior to the addition of the detergent. Many commercial formulations are available for this procedure.
2. Fixation. Typical fixatives include formalin, paraformaldehyde, or an alcohol such as methanol. The goal of the fixation is to maintain cell integrity without being so harsh as to destroy the epitopes detected by the antibodies. A weak formalin solution is used most often.
3. Permeabilization. This is accomplished by using a detergent such as saponin, triton X-100, NP-40, or tween 20.
4. Intracellular antigens evaluated routinely by flow cytometry include terminal deoxynucleotide transferase (TDT) and myeloperoxidase (MPO) in acute leukemia samples and immunoglobulin light chains in plasma cell disorders.

(b) Instrument

(i) Flow cytometers require three systems:

1. A fluidics system to arrange cells linearly as they pass the laser.
2. An optic system to project the laser light onto the cells with a set of filters and mirrors directing the emitted light to appropriate detectors.
3. An electronic system to convert the detected light into electronic signals.

(ii) The fluidics system in a flow cytometer forces cells in the sample into single file allowing for sequential one-by-one interrogation. The process is accomplished by *hydrodynamic focusing* which uses velocity differences between two fluid streams to direct the flow of cells suspended in a liquid, Fig. 22.2. The sample is put into a flow stream surrounded by a second liquid layer running at higher velocity although at lower pressure. The fluids will not intermix if matched for viscosity and hydrophobicity. The surrounding liquid layer is referred to as *sheath fluid* and directs the flow of the sample fluid. The width of the sample fluid is dependent on pressure differences between the two liquids. Reducing the width of the sample fluid by lowering the pressure gradient forces cells into the linear arrangement necessary for analysis of cells one by one. The speed of the fluidics system is extremely fast. In a typical analysis, 100,000 or more cells are collected, a process that may take no more than a few seconds.

(iii) The optical system

1. Lasers. Lasers produce a single wavelength of light and are used to excite the fluorochromes detected by the instrument.
   (a) Due to the properties of fluorescence, usually only 3–5 fluorescent compounds can be excited and subsequently detected from a single laser wavelength of light.
   (b) To increase the number of fluorochromes detected, additional lasers have been added to instruments.
   (c) Current cytometers generally have three or four lasers and are capable of detecting eight or more fluorescent molecules simultaneously. Samples requiring interrogation by more molecules, typically necessary in clinical practice, require multiple-stained tubes.
   (d) The number of different fluorescent compounds that can be detected by a flow cytometer is referred to as the "colors" of the instrument. A flow cytometer that can detect six fluorescent compounds is considered to be a "six-color" instrument.
2. Filters. Filters are placed in the optical path after light is emitted to detect the wavelengths of light corresponding to the fluorochrome.
   (a) Fluorochromes do not emit at a single wavelength of light but over a spectrum of light wavelengths. Filters usually limit light that is captured to a specified bandwidth (e.g., 520–550 nm).
   (b) Light emitted from one fluorochrome is often not restricted to the bandwidth set for its detection but may also fall into the filter bandwidth set for another fluorochrome. This "spillover" light must be removed electronically, a process referred to as *compensation*. Failure to remove spillover light results in false-positive signals in the "spillover" channel, Fig. 22.3.
   (c) Light is also detected in the forward direction relative to the incoming laser light. This is referred to as *forward angle light scatter (FSC)* and is roughly proportional to cell size.
   (d) Light scattered at 90° angle to the incident laser light is referred to as *side angle light scatter (SSC)*. SSC reflects cytoplasmic complexity of the cell, usually as the result of granulation.

(iv) Electronics

(a) Detection. Light signals (photons) are detected and converted to electronic signals by a photomultiplier tube (PMT) for data display. A PMT transforms photons into a

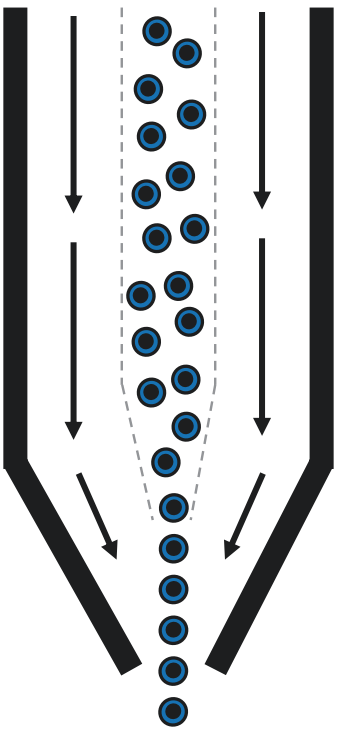

Fig. 22.2 Hydrodynamic focusing

proportional number of electrons creating an electrical current which is then converted to a voltage pulse and recorded. Since instruments are now digitized each cell is given values corresponding to the voltage pulse for each fluorochrome, FSC and SSC. For instance, each cell probed on a six-color instrument will have eight numbers associated with it, one for the intensity of light emitted from each fluorochrome ($n = 6$), FSC, and SSC. On such an instrument, the data generated is eight dimensional.

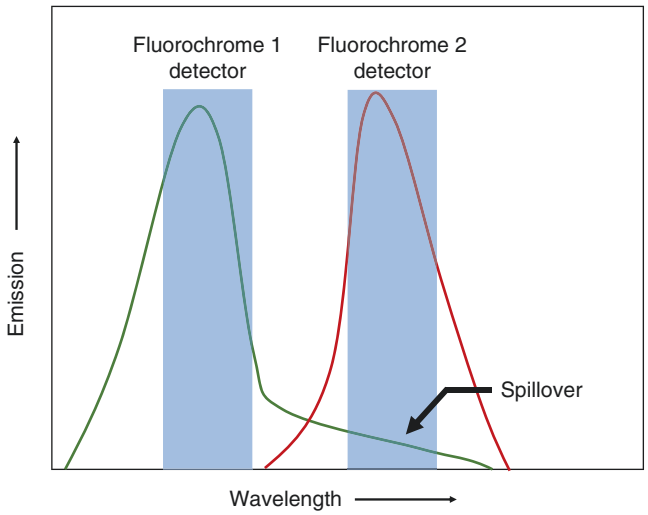

Fig. 22.3 Fluorescent "spillover"

(b) Integration of data. For multi-laser instruments, cells pass by each laser sequentially rather than simultaneously. Therefore, the emitted light data collected from any one individual cell is not detected at the same time. A method is needed to match the data collected from each cell as it passes by successive lasers. This is accomplished by *time delay integration*. Simply, the time interval between which the cell passes through each laser beam is known so that all parameters collected for each cell can be reintegrated and processed together simulating a single event.

(v) Data display. Although the data generated is highly dimensional, it is typically visualized in one or two dimensions: single-dimension *histogram plots* and two-dimensional *dot plots*, Fig. 22.4.

1. *Histogram plots*. A histogram plot is a plot of fluorescent intensity (*x*-axis) versus cell number (*y*-axis). These plots are useful to quantify the mean fluorescent intensity of a bound fluorescent analyte or determine the percentage of cells that express an antigen. An example is the quantification of CD59 expression in paroxysmal nocturnal hemoglobinuria (PNH), Fig. 22.4.
2. *Dot plots*. These are graphs where each dot represents a cell. The plots are used frequently and display fluorescence intensity of one fluorochrome versus another, Fig. 22.4.

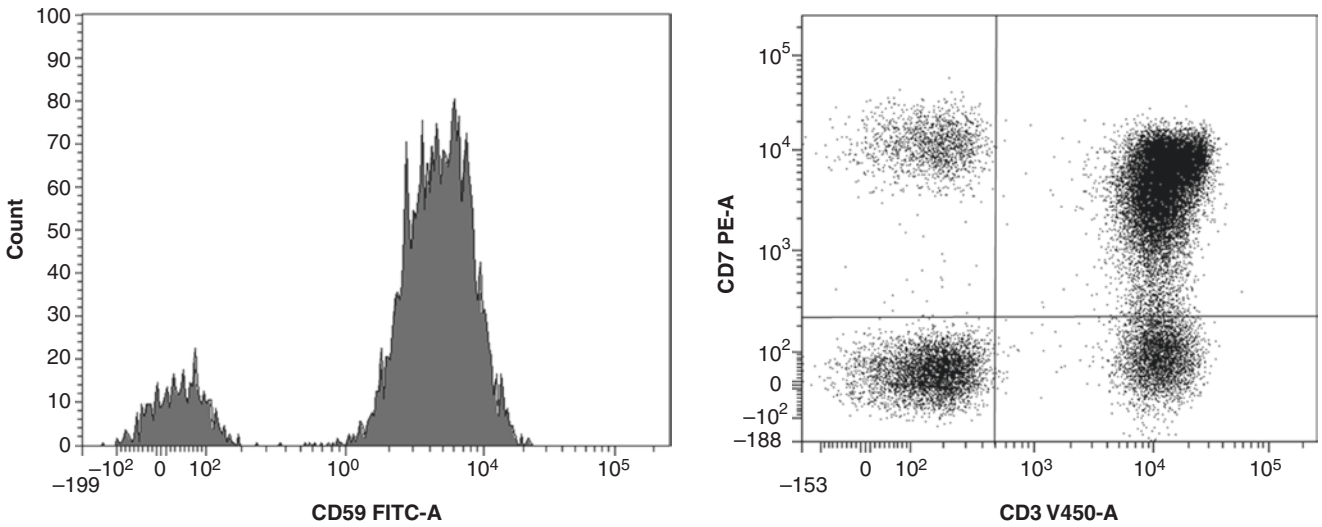

Fig. 22.4 Histogram and dot plots. In a histogram plot, left, intensity of an antigen is expressed on the *X*-axis and cell number on the *Y*-axis. A histogram plot for CD59 is shown. Red cell expression of CD59 is used for the evaluation of paroxysmal nocturnal hemoglobinuria. For a dot plot, right, intensity of two analytes is plotted against another. CD3 vs CD7 plot is useful to evaluate for a T cell neoplasm

Dot plots are the best means to visualize data from samples suspected of harboring neoplasia.

(a) Multiple dot plots are necessary to examine the data generated. For an instrument of "*n*" colors, $(n^2 - n)/2$ dot plots are required for all fluorochrome vs fluorochrome comparisons. This means for an eight-color instrument, $(8^2 - 8)/2 = 56/2$ or 28 dot plots would be required for each analytic tube that is set up. In reality, not all potential graphs need to be represented as the information may be redundant.

(b) Comparison of one analyte to another on a population of cells typically results in four different cellular groups, X(+) Y(−), X(+)Y(+), X(−)Y(−), and X(−) Y(+), although careful examination of the data often reveals other populations based on staining intensity level. An example is shown for lymphocytes stained with CD3 and CD7, Fig. 22.5.

(c) Fluorescent background. All cells have some level of fluorescence, referred to as *autofluorescence*, even in the absence of added fluorescent probes. This baseline fluorescence must be determined using unstained cells or controls with non-cell binding fluorescent-conjugated antibodies. Any fluorescence detected above this level when a fluorescent

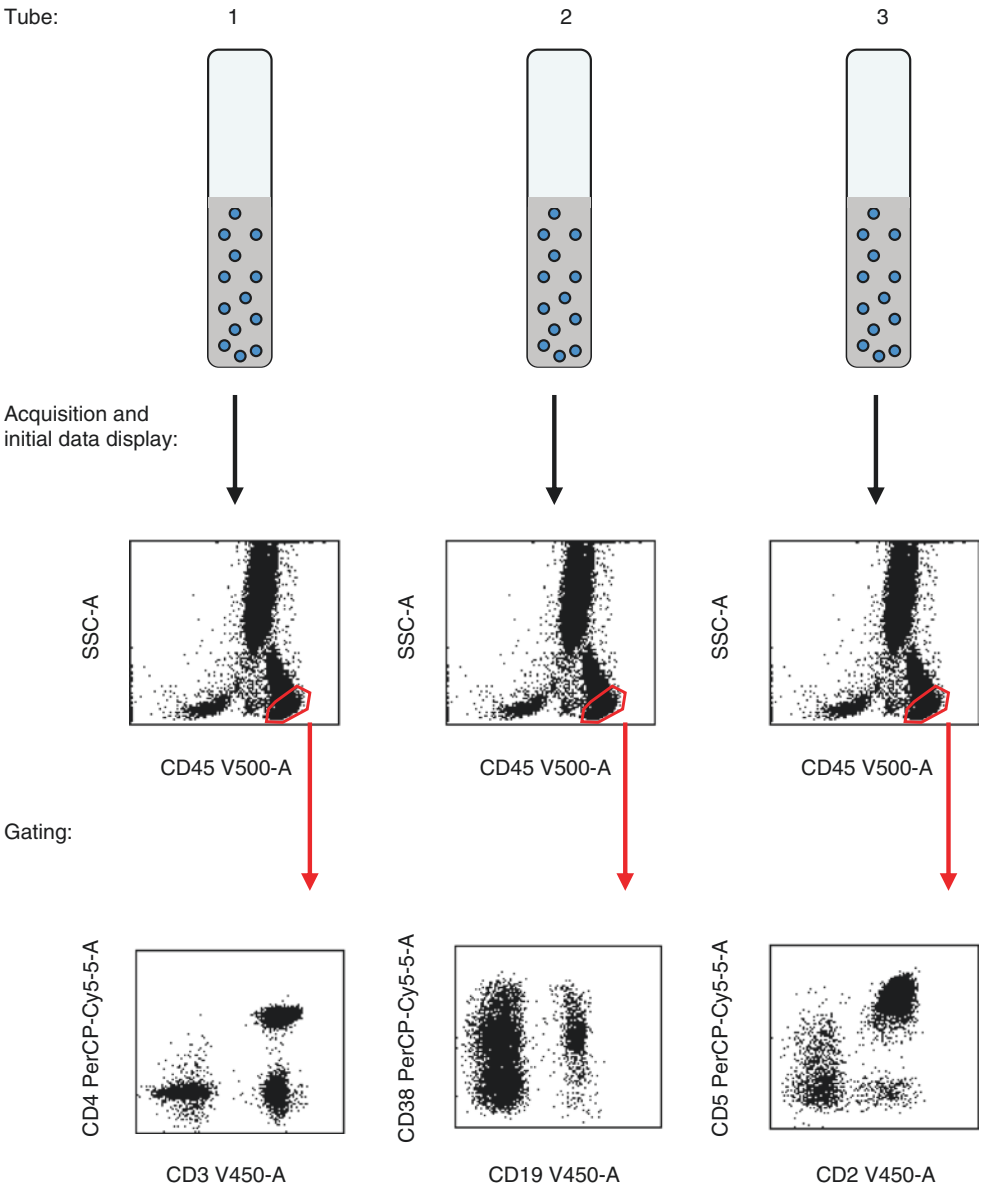

Fig. 22.5 Gating. Data from cells acquired from one tube can be correlated with those collected from another as long as one or more parameters are identical

probe of known specificity is added can safely be attributed to binding of the probe to the cell. Positive cutoff lines based on the baseline fluorescence are added to graphs to aid in visual evaluation.

3. *Gating*. Gating is the process of electronically selecting cells based on their fluorescent and light scatter characteristics, Fig. 22.5.
   (a) Gating is needed when information must be gleaned from a specific cell subset rather than the population as a whole.
   (b) Since most clinical analyses require multiple-stained tubes, it becomes important to link cell populations observed in one tube with that seen in others. The cells being analyzed are not the same, but the populations they represent are. For instance, cells in the low FSC × SSC region represent lymphocytes. Maintaining the same gated region between tubes ensures that lymphocytes of the same character and composition are evaluated.
   (c) For comparison of gated cell populations between tubes, the same parameter must be maintained between tubes. This can be a specific fluorochrome-conjugated antibody and/or FSC and SSC. FSC and SSC information is always collected and is a useful aid in gating.
      (i) FSC vs SSC plots. Data from light scatter alone can separate the three main white cell populations in peripheral blood: lymphocytes, monocytes, and granulocytes, Fig. 22.6. However, a FSC vs SSC plot does not allow discrimination of more complex mixtures of cells as may be present in bone marrow. Nucleated red cells and lymphocytes have comparable light scatter characteristics and cannot be resolved using FSC and SSC alone.
      (ii) CD45 VS SSC plots. CD45, also known as leukocyte common antigen, is expressed on all white blood cells but not on nucleated red cells. CD45 vs SSC plots are superior to FSC and SSC graphs for separating the main leukocyte populations for gating. Most of the main cell populations in blood and bone marrow are resolved using CD45 and SSC plots, Fig. 22.6. For these reasons laboratories commonly add CD45 to all stained tubes to facilitate gating in these specimens.
      (iii) Identifying cell subsets based on antigen expression. For example, CD3 can be added to all tubes to facilitate gating of T cells since surface CD3 expression is a defining feature of mature T cells. Gating on CD3-expressing cells ensures the evaluation is restricted only to T cells.

C. Advantages. The strength of flow cytometry resides in its sensitivity and multi-parametric ability. It has several advantages compared to tissue-based antigen detection (immunohistochemistry, IHC).
   (a) Multi-parametric ability. As indicated flow cytometers are capable of detecting many colors simultaneously. This enables the identification of an abnormal cell population within a complex cell mixture such as bone marrow specimens. The simultaneous assess-

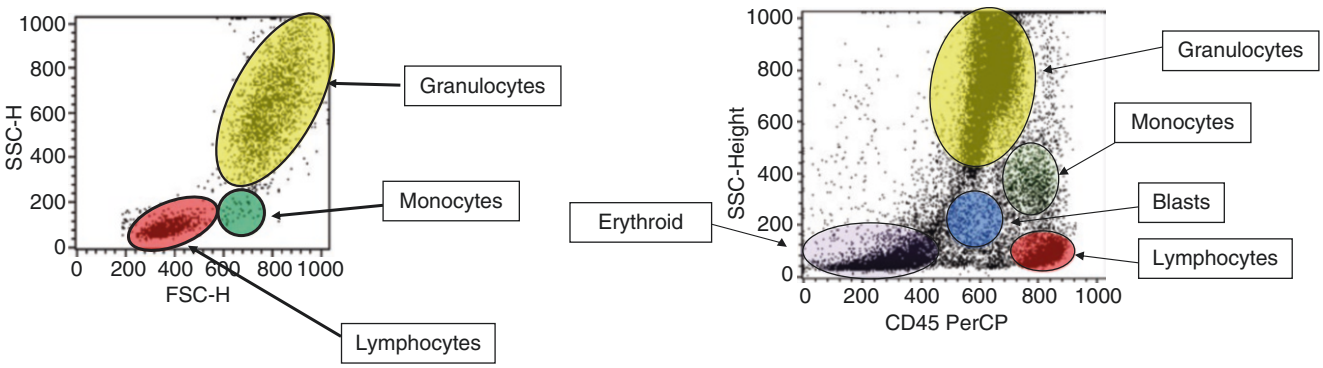

Fig. 22.6 Gating based on light scatter and CD45. FSC vs SSC plot (left) and CD45 vs SSC plot (right)

ment of "*n*" antigens segregates cells into 2^n non-overlapping cell subsets. For a 4-color instrument ($n = 4$), 16 separate cell populations can be resolved. IHC stains also detect expressed antigens on cells; however IHC is essentially limited to evaluating one antigen at a time impeding the ability to determine the relationship of the antigens detected.

(b) Sensitivity. Flow cytometers, as mentioned previously, can detect roughly 100 molecules per cells making them significantly more sensitive than IHC stains on an antigen per cell basis.

(c) Quantitative. The fluorescent data is acquired is quantitative and objective.

(a) The amount of fluorescent probe bound per cell is proportional to the fluorescence detected. Therefore, differences in expression can be quantitated. Although this is rarely necessary in clinical practice, the ability to easily visualize subtle differences in antigen expression between cell populations greatly enhances the sensitivity of detection of abnormal cells by flow cytometry compared with IHC.

(b) Cell populations can be quantitated. Not only expression levels but distinct cell populations (e.g., CD3+CD4+ cells as a percentage of lymphocytes) can be easily quantitated. Flow cytometry became integrated into the clinical laboratory in the early 1980s initially as a means to quantitate CD4 cell counts as the HIV/AIDS epidemic was unfolding.

(d) Objectivity. Tissue sections stained with antibodies using IHC require examination under a microscope and subjective assessment. Flow cytometry data is evaluated objectively.

(e) Ease of use. Current instruments are easy to operate and many functions are automated. Interpretation of data does require expertise and knowledge, however.

D. IHC vs flow Cytometry overview

(a) Historically, many more antibodies were available for flow cytometry than for IHC. Flow cytometry was therefore necessary to adequately phenotype cells from tissues.

(b) Antigens are denatured when tissues are placed in formalin, the most common fixative used for histology. The alteration of antigen structure often results in loss of epitopes that are recognized by antibodies generated using antigens in their native state. Most antibodies were originally developed this way, limiting the ability to detect antigens in fixed tissue sections. However, in the last two decades, numerous antibodies have been created to epitopes on antigens that remain after formalin fixation greatly expanding the ability to detect antigens in tissue sections. Additionally, automation and staining technology advancements have helped expand the repertoire of reagents for IHC.

(c) Intracellular epitopes in some instances are more amenable to detection by IHC than flow cytometry.

(i) As mentioned, special intracellular staining techniques are required to probe for antigen expression inside a cell by flow cytometry. IHC staining by its nature evaluates antigen expression both inside the cell and on the cell surface. IHC is performed on thinly cut tissue sections (~4 μ) placed on glass slides. The sectioning results in slicing a cross-section through cells allowing access to the cell interior. Therefore, staining differences between flow cytometry and IHC may result depending on the location of an antigen. For instance, progenitor T cells partially express the CD3 molecular complex and retain one of the CD3 chains, CD3ε, intracellularly until the full complex can be synthesized. As a result, staining of these cells for CD3ε by flow cytometry using standard methods reveals no or only partial reactivity; however IHC staining is positive in all cells.

(ii) Some intracellular molecules detected by IHC cannot be assessed by flow cytometry. For unclear reasons some antigens can be detected after formalin fixation and tissue sectioning but not by standard flow cytometry in their "native" state. The most relevant clinical example is cyclin D1, a protein dysregulated in mantle cell lymphoma. Simple flow cytometry methods for its detection have not been successful although it is easily stained by IHC.

(d) The advent of antibodies that work well in formalin-fixed tissue sections reduces the importance of phenotyping by flow cytometry. However, flow cytometry remains critical for the evaluation of tissue biopsy material for two reasons:

(i) Documenting clonality through light chain expression.

(ii) Identifying neoplastic cells. Hematopoietic tumor samples contain an admixture of both benign reactive elements and neoplastic cells. When reactive cells are numerous, evaluation by single parameter IHC can be very difficult. Also, not uncommonly, a sample may contain more than one neoplastic process which may be extremely difficult to appreciate by IHC. The multi-parametric ability of flow cytometry becomes critical in identifying and phenotyping the neoplastic cell population(s) in these situations.

(e) Surface light chain expression cannot be detected by IHC using standard techniques likely due to the low-level expression of these antigens. Therefore, flow cytometry is necessary for the documentation of clonality in tissue biopsy specimens. Most surgeons are well aware of the need to procure fresh tissue for flow cytometry whenever a hematopoietic neoplasm, particularly a B cell lymphoma, is suspected.

(f) Importantly, *flow cytometry is the technique of choice when evaluating cells from liquid tissues such as blood and bone marrow*.

E. Limitations

(a) Flow cytometry is not particularly useful for the detection of solid, non-lymphoid neoplasms since cells need to be dispersed when analyzed. Carcinoma cells contain tight junctions between cells preventing adequate cellular dispersal.

(b) Large cells and cells adherent to other elements, such as carcinoma cells, Reed-Sternberg cells in Hodgkin lymphoma, and plasma cells are poorly identified by this technique. Otherwise, the relative cell numbers seen by flow cytometry are roughly equivalent to that on marrow aspirate smears.

(c) Elements that may not aspirate well, such as cells in lymphoid aggregates in a bone marrow, are often underrepresented when examined by flow cytometry. The actual extent of marrow involvement by these processes is therefore best determined on the bone marrow core biopsy.

(d) Flow cytometry does not incorporate visual data and therefore should be considered complementary to and not used in place of morphology.

(e) Viable cells are needed. Fresh samples are required. Samples that may have a prolonged transit time to the laboratory or are stored prior to analysis may affect the evaluation. Generally, blood, bone marrow and lymph node samples can be stored for 24–48 h without impairing sample integrity. Some analytes and cell subsets, however, are prone to degradation in vitro after 24 h.

(f) No standard volume of fluid is aspirated; therefore absolute counts cannot easily be determined from the instrument.

F. Applicability

(a) Flow cytometry is warranted in most instances when a hematopoietic neoplasm is suspected in blood, bone marrow or tissue biopsy or to monitor disease in the blood or bone marrow after therapy. Clinical conditions in which flow cytometry should be performed are indicated in Table 22.1. Aspirated bone marrow should be sent for flow cytometry in most instances.

Table 22.1 Flow cytometry in hematopoietic neoplasia

Indicated
Cytopenia
Elevated leukocyte count
Lymphocytosis
Monocytosis
Eosinophilia
Atypical cells or blasts on smear or in body fluid
Plasmacytosis or evaluation of monoclonal gammapathy
Organomegaly, lymphadenopathy, or tissue masses
Dermatologic disorder
Patient monitoring
Staging of lymphoma
Residual disease after therapy
Detection of specific therapeutic targets
Documentation of progression or relapse
Diagnosis of a secondary neoplasm (therapy-related neoplasm)
Evaluation of disease acceleration
Prognostication (e.g., ZAP-70 in CLL)
Not indicated
Mature neutrophilia
Polyclonal hypergammaglobulinemia
Polycythemia
Thrombocytosis
Bone marrow staging for Hodgkin lymphoma
Routine monitoring of a myeloproliferative neoplasm

Table 22.2 Flow cytometry testing in hematology

Disease/process	Classic method	Flow method	Target
Hereditary spherocytosis	Osmotic fragility	Eosin 5′maleamide	Band 3 protein
Fetal-maternal hemorrhage	Kleihauer-Betke	Anti-hemoglobin F	Fetal red cells
Paroxysmal nocturnal hemoglobinuria	Ham test	Anti-CD59, FLAER	GPI-anchors

(b) Benign hematology

(a) Flow cytometry has replaced several tests used in hematology to identify pathologic non-neoplastic hematologic processes due to its ease of use, sensitivity, and reliability, Table 22.2.

(b) Paroxysmal nocturnal hemoglobinuria (PNH). Flow cytometry is the technique of choice in the evaluation of patients for PNH. PNH is a rare disease that results from an acquired mutation in the hematopoietic stem cell leading to disrupted synthesis of glycosyl-phosphatidyl-inositol (GPI) moieties. GPIs are glycolipid structures that are covalently attached to some cell surface proteins and serve to anchor these polypeptides to the cell membrane much like a string to a balloon when affixed to a solid support. If the string is lost, the balloon floats away. So too

when the GPI structure is not synthesized, GPI-anchored proteins are lost from the cell membrane. Assaying for the absence of any one of the GPI-anchored molecules allows for the identification of cells affected by the process. Typical antigens assessed include CD59 on red cells and CD14 and CD24 on white cells, Fig. 22.4 (left). A bacterial protein aerolysin has been found to bind to the GPI structure, and a chemically modified fluorescent form has been produced known as *FLAER or fluorescent aerolysin*. It is also useful to detect GPI-deficient cells in PNH.

(c) Enumeration of lymphoid cell subsets. The determination of blood levels of the main lymphoid subsets including CD4 and CD8 T cells, natural killer cells, and B cells is performed by flow cytometry. Lymphoid subset evaluation is performed in individuals suspected of having a primary or secondary immunodeficiency or to monitor cell subsets that may be affected by immunotherapy such as after treatment with the anti-CD20 antibody, rituximab targeting B cells. Rituximab may cause loss of B cells from the peripheral blood and tissues for prolonged periods (months). The effects of the drug can be tracked by examining blood samples using a second B cell-specific antibody such as CD19. The latter ensures that B cells are detected even in the presence of rituximab since CD20 may be masked by the reagent not allowing for detection by flow cytometry when anti-CD20 antibodies are utilized. More detailed analysis of lymphoid cells may be necessary for the evaluation of an immunodeficiency although these are generally performed in specialty laboratories, Table 22.3.

(d) Stem cell enumeration. Determination of the number of hematopoietic stem cells is critical for patients undergoing hematopoietic stem cell transplantation. Stem cells capable of re-populating the bone marrow reside in the CD34+ cell fraction. As such, the total number of CD34+ cells infused into a patient is a main determinant of marrow recovery after hematopoietic cell transplant. Therefore CD34+ cells are enumerated in all products used for hematopoietic stem cell transplant. Additionally, CD34+ cells are collected from peripheral blood, cord blood, or bone marrow prior to transplantation. Assessing the number of cells in these specimens is necessary to determine their adequacy as a source of hematopoietic stem cells.

(a) Method. The CD34 enumeration method is standardized in clinical laboratories. The *I*nternational *S*ociety of *H*ematotherapy and *G*raft *E*ngineering, ISHAGE, protocol is used. The method requires the use of a CD45 and CD34 antibody with a specified gating sequence, Fig. 22.7.

(b) Viability. The hematopoietic stem cells used for transplant must be viable for a useful graft. CD34 enumeration methods incorporate fluorescent viability dyes to assess cell viability. All viability methods use fluorescent dyes that bind nucleic acids (RNA and DNA) but cannot penetrate an intact cell membrane. When the cell membrane is disrupted as part of cell death, the dye gains access to the cell interior and binds nucleic acids resulting in a fluorescent signal. Typical viability dyes include propidium iodide and 7-aminoactinomycin D.

(c) Absolute counting. As mentioned, flow cytometers do not aspirate a fixed amount of fluid and are incapable of determining cell concentration. Only relative cell percentages are enumerated. Cell concentrations can be calculated by multiplying the percentage of cells identified by flow cytometry by the white count determined on a standard hematology analyzer. This *dual platform method* of cell enumeration is adequate for almost all clinical purposes except for CD34+ cell counting in specimens used for hematopoi-

Table 22.3 Flow cytometry in immunodeficiency

Disease	Analyte
Autoimmune lymphoproliferative disorder	% CD4-CD8- T cells
Hyper-IgM syndrome	CD40, CD40 ligand
HYPER-IgE syndrome	% TH17 cells, STAT3, DOCK8
Chronic granulomatous disease	Neutrophil oxidative burst
Common variable immunodeficiency	ICOS, BAFF, naïve/memory B
Neutrophil adhesion deficiency	CD18, CD11A
Bare lymphocyte syndrome	MHC CLASS I and II
Immunodysregulation, polyendocrinopathy	FOXP3, regulatory T cells
Enteropathy, X-linked (IPEX) syndrome	
Hemophagocytic lymphohistiocytosis	Natural killer cell degranulation
	Perforin, XIAP, SAP
X-linked lymphoproliferative disorder	% invariant NKT cells
	XIAP, SAP
Severe combined immunodeficiency	B and T subsets, naïve/memory T
	ZAP-70

ICOS inducible T cell costimulator, *BAFF* B cell activating factor, *XIAP* inhibitor of apoptosis, X-linked, *SAP* slam-associated protein

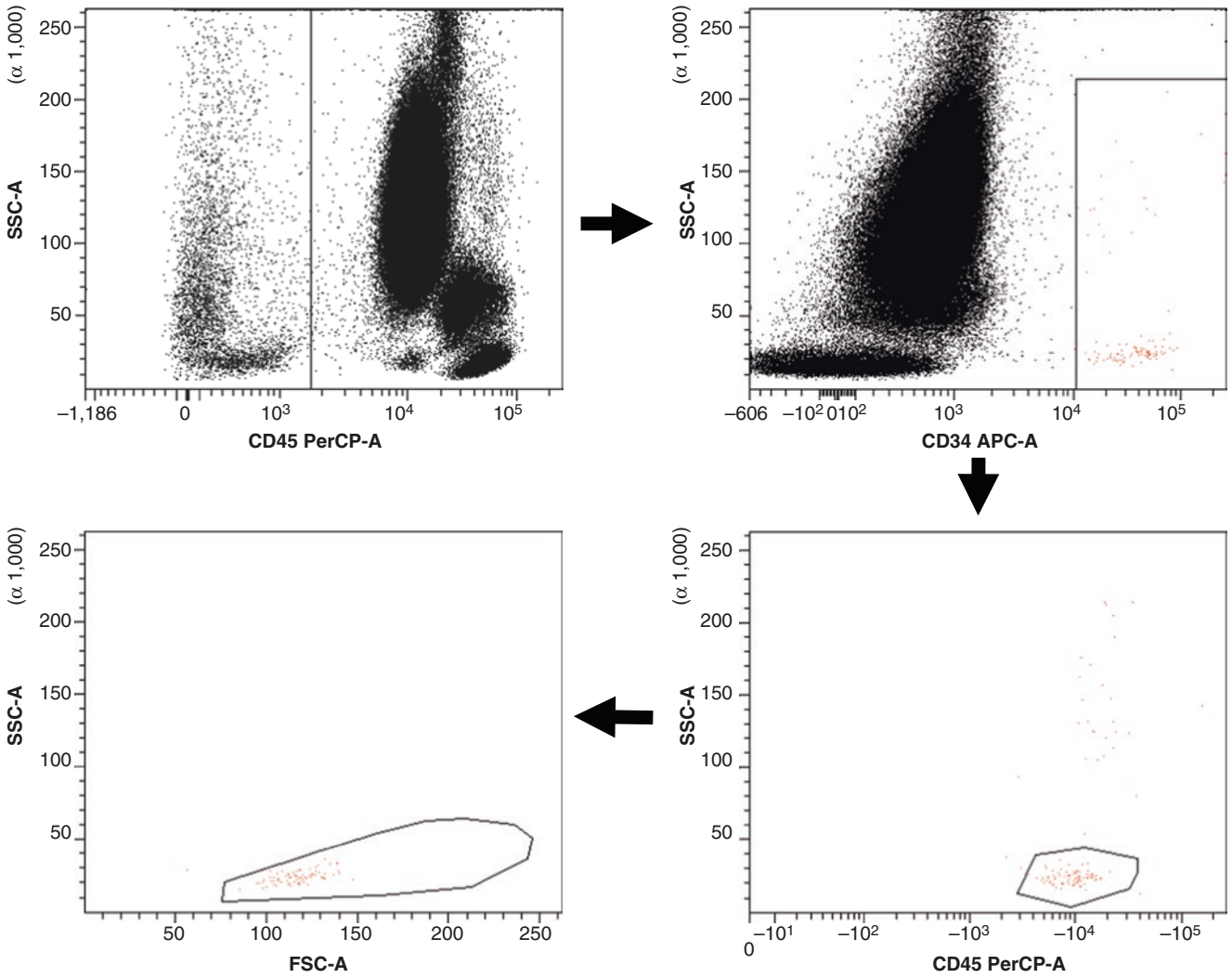

Fig. 22.7 ISHAGE method for enumerating CD34 hematopoietic stem cells

etic transplantation. This is because poorly viable white cells within a product may be counted on a hematology analyzer but lost during cell processing prior to assessment on a flow cytometer resulting in an artificially high CD34+ cell percentage. To circumvent this problem, a known quantity of beads is added to a specified volume of sample while evaluating CD34+ cells by flow cytometry. Counting the number of beads collected on the instrument allows for determination of the volume aspirated and the concentration of CD34+ cells can be directly determined. This is the basis for *single platform method* of CD34+ cell enumeration, Fig. 22.8.

(e) Detection of allogeneic antibodies. Allogeneic antibodies are important in solid organ transplant. Preformed antibodies in the host to a donor's human leukocyte antigens (HLA) are associated with early rejection and accelerated graft loss. Flow cytometry is used to detect these antibodies using a fluorescent version of the indirect antiglobulin test referred to as the *flow cytometry crossmatch*. Fluorescent-conjugated antibody to human IgG is used to detect antibodies that bind a donor's T lymphocytes (anti-HLA class I antibodies) and B lymphocytes (anti-HLA class I and/or HLA class II antibodies) after incubation with the host's serum.

(f) Flow cytometry testing for platelet disorders

(a) Immune thrombocytopenia. Autoantibodies to platelets are felt to be the cause of immune thrombocytopenia. Flow cytometry tests can be performed to help detect these antibodies. Patient serum is tested for reactivity to a pool of normal platelets and detected using fluorescent-conjugated antibody to human IgG and IgM.

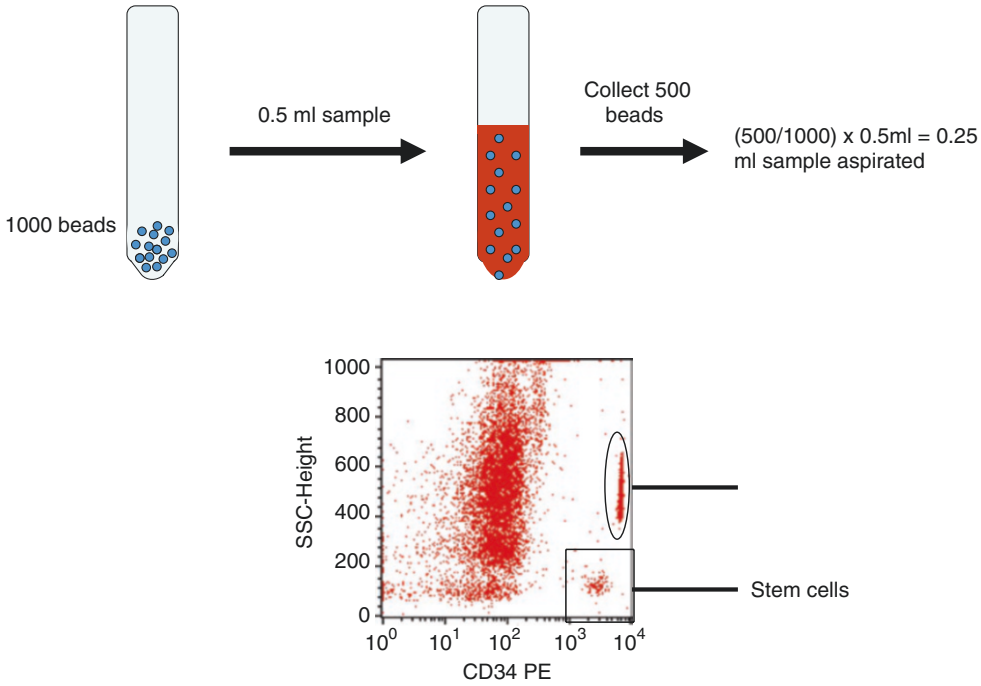

Fig. 22.8 Single platform method for determining concentration of CD34 hematopoietic stem cells. Staining method is shown at the top. Beads and cells are counted on flow cytometer (bottom)

(i) The method is superior to other lab tests for detecting antiplatelet antibodies when probing for low-density or labile antigens.

(ii) Limitations

1. If positive, the test does not identify the specific antibody target.
2. Specificity is poor since reactivity may due to anti-HLA antigens or other antigens not restricted to platelets.

(b) Diagnosis of inherited platelet disorders. Congenital lack of platelet glycoproteins GPIIb/IIIa ($\alpha_2\beta_3$ integrins) and GPIb leads to the inherited bleeding disorders Glanzman's thrombasthenia and Bernard-Soulier syndrome, respectively. The presence of the antigens can be assessed on the platelet surface by flow cytometry to identify these disorders.

(c) Platelet function testing

(i) Novel protein structures and lipids are expressed on the surface of activated compared to resting platelets and can be assayed to assess platelet function in vivo or after stimulation in vitro. These include:

1. P-selectin (CD62p)
2. PAC-1 which recognizes the unique activation conformation of GPIIb/IIIa complex.
3. Annexin V which binds the inner lipid bilayer constituent phosphatidylserine which becomes exteriorized on an activated platelet.

(ii) Mepacrine (quinacrine) uptake. The fluorescent reagent is taken up by platelet dense granules and can be used to detect platelet dense granule deficiency.

G. Methods of assessment in neoplasia

(a) Neoplastic cells can be identified by flow cytometry based on quantitative changes in cell subset distribution or qualitative alterations in antigen expression.

(b) In general, quantitative changes are less reliable for the identification of neoplasia than qualitative aberrancies.

(a) Quantitative changes. Examples include:

(i) Skewed kappa/lambda light chain distribution in B cell lymphoma, Fig. 22.9.

(ii) Increase percentage of CD7-CD4+ T cells in T cell lymphoma, Fig. 22.10.

(iii) Increase in CD34+CD117+ cells (myeloblasts) in myelodysplastic syndrome, a preleukemic state, Fig. 22.11.

(b) Qualitative changes, examples include:

(i) Atypical expression of an antigen not normally expressed by the cell type such as CD5 expression B lymphocytes in chronic lymphocytic leukemia, Fig. 22.12.

(ii) Abnormal antigen expression compared to normal during cell development such as CD10 and CD20 in B cell lymphoblastic leukemia/lymphoma (see Fig. 22.19).

(iii) Abnormal expression level of an antigen. For example, reduced CD3 expression in T cell neoplasms, Fig. 22.10.

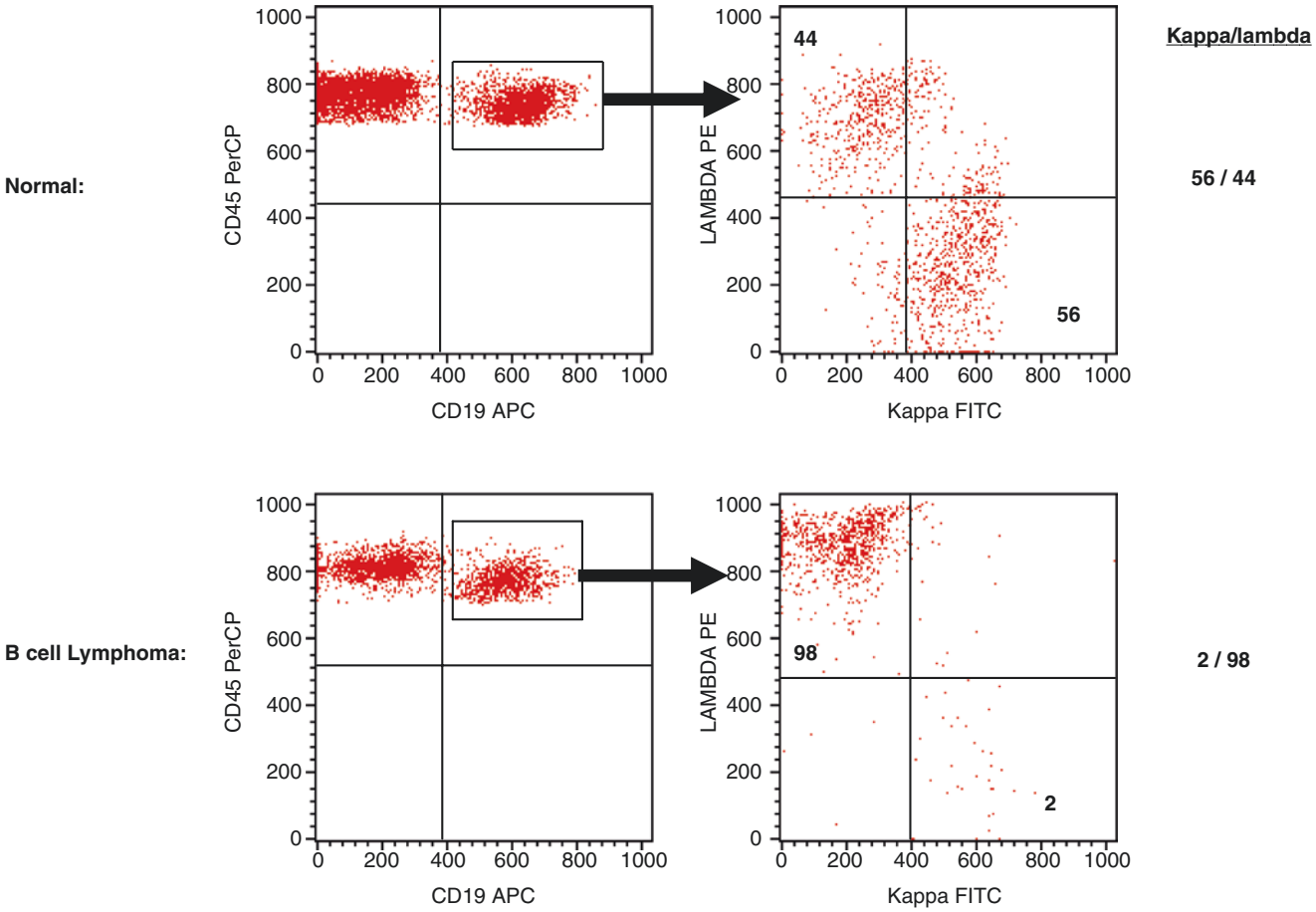

Fig. 22.9 B cell clonality. The ratio of kappa and lambda light chain expressing B cells can be used to determine clonality of B lymphocytes

(c) Atypical antigen expression is key to identifying neoplastic cells. A detailed knowledge of normal antigen expression on hematopoietic cells is critical for the evaluation of flow cytometry graphs when evaluating for malignancy. Examples of the antigen expression changes during cell maturation for B cells and granulocytes are shown in Figs. 22.13 and 22.14, respectively. Alterations from normal strongly suggest neoplasia.

(d) Examples

(a) Clonality of B cells as an indicator of B cell lymphoma. Clonality equates with neoplasia in most instances. B cells fortuitously offer an easy means to identify clonality. Mature B cells express immunoglobulin molecules on their surface composed of a heavy chain and one of two light chains, kappa and lambda. Importantly, only a single type of light chain is incorporated into an immunoglobulin and expressed on any particular B cell due to *allelic exclusion.* In humans this results in a kappa/lambda light chain ratio on a population of mature B cells of roughly 3:2. Clonal expansion of a B cell population alters the light chain ratio since allelic exclusion is maintained in B cell tumors. A ratio of more than 5:1 toward kappa and 2:1 toward lambda indicates the presence of a clonal population of B cells, Fig. 22.9. As a result, clonality of B cells is easily determined by flow cytometry when B cells are stained with antibodies to light chains.

(i) In practice, all lymph node samples and samples sent to evaluate for a lymphoproliferative process in blood, bone marrow, or a body fluid are stained with antibodies to the light chains in combination with a B cell marker such as CD19 and CD20. B cells are gated and the light chain ratio determined.

(ii) B cell progenitors have not fully rearranged their immunoglobulin genes and therefore do not express light chains. Therefore, clonality cannot be determined on these cells using this technique.

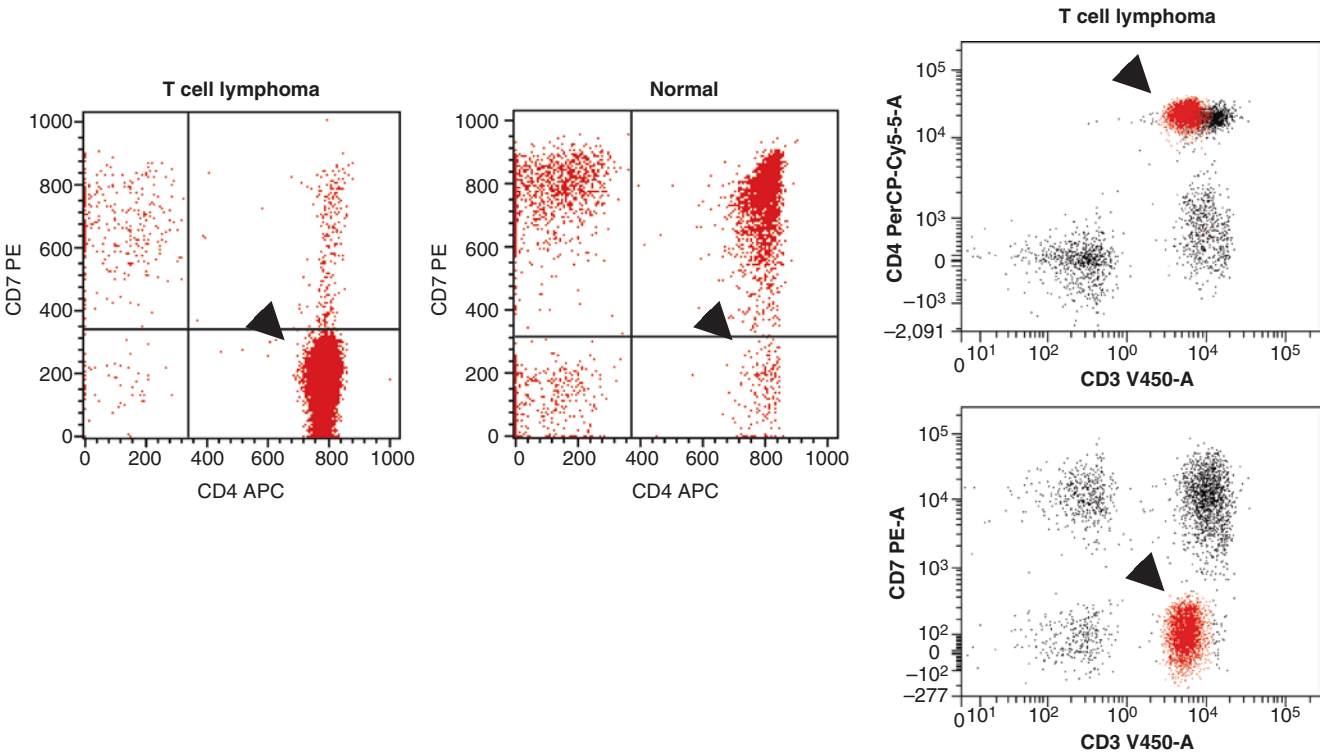

Fig. 22.10 Identifying T cell neoplasia. Quantitative changes such as increase in CD7- CD4+ T cells, left, suggest a T cell clonal process. Qualitative alterations, for example, reduced expression of CD3, shown at right, are a more specific means to identify a T cell malignancy. Example at right, from a patient with mycosis fungoides/Sezary syndrome, demonstrates both abnormalities

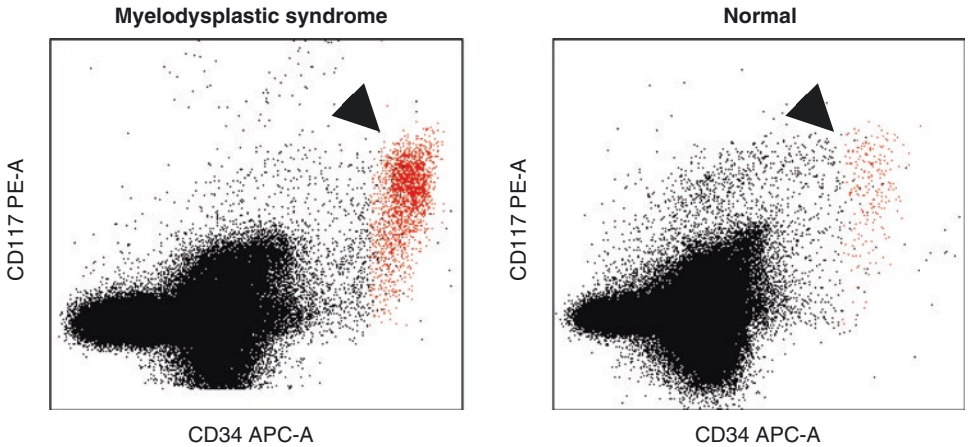

Fig. 22.11 CD34+CD117+ myeloblasts. Myeloblasts, defined as CD34+CD117+ cells, are commonly increased in myelodysplastic syndromes

(iii) Cells from some mature B cell neoplasms may not express light chains as an aberrancy. Clonality in these situations can be inferred by the atypical lack of light chains in a population that normally should have expression.

(iv) Plasma cells are the end stage of B cell maturation. These cells change their expression of immunoglobulin from a cell surface-associated form to a secreted form causing loss of surface expression of immunoglobulins, including light chains. As a result, clonality cannot be assessed by surface light chain staining. However, the immunoglobulin molecules are present within the cytoplasm of the cells prior to secretion and can be detected using intracellular staining. Similar to mature B cells, an abnormal light chain ratio of intracellular immunoglobulins indicates the presence of a clonal process, and neoplasia can be inferred.

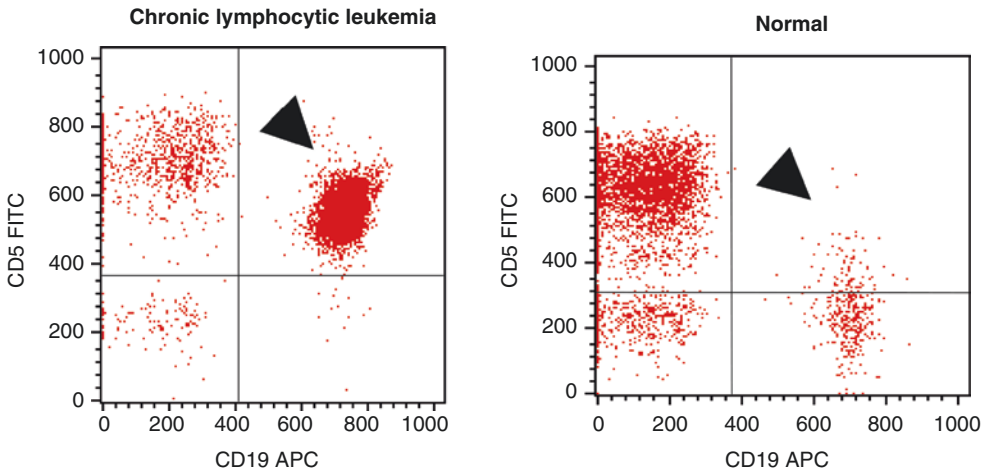

Fig. 22.12 CD5 expression on B cells in chronic lymphocytic leukemia. CD5 is normally only weakly expressed on a subset of normal B cells but is strongly and uniformly expressed on B cells in chronic lymphocytic leukemia

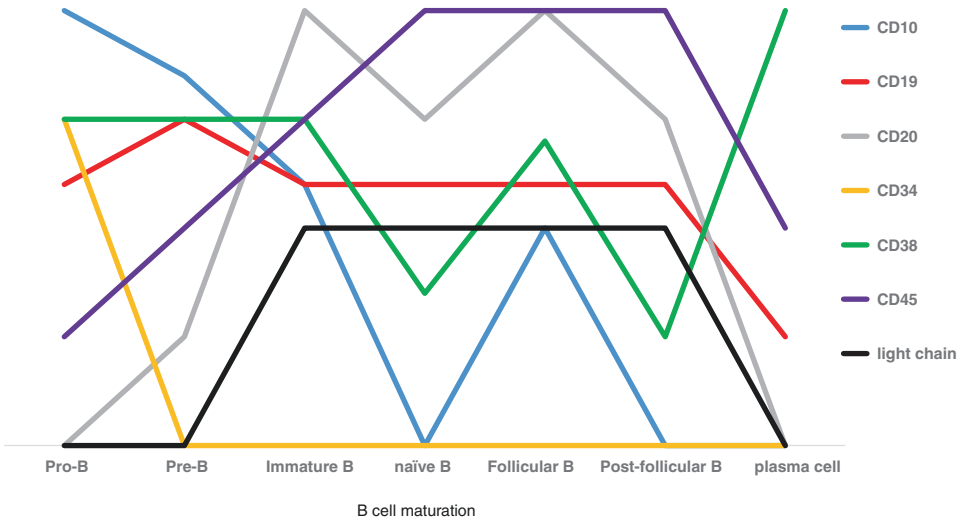

Fig. 22.13 Changes in expression of antigens during B cell development

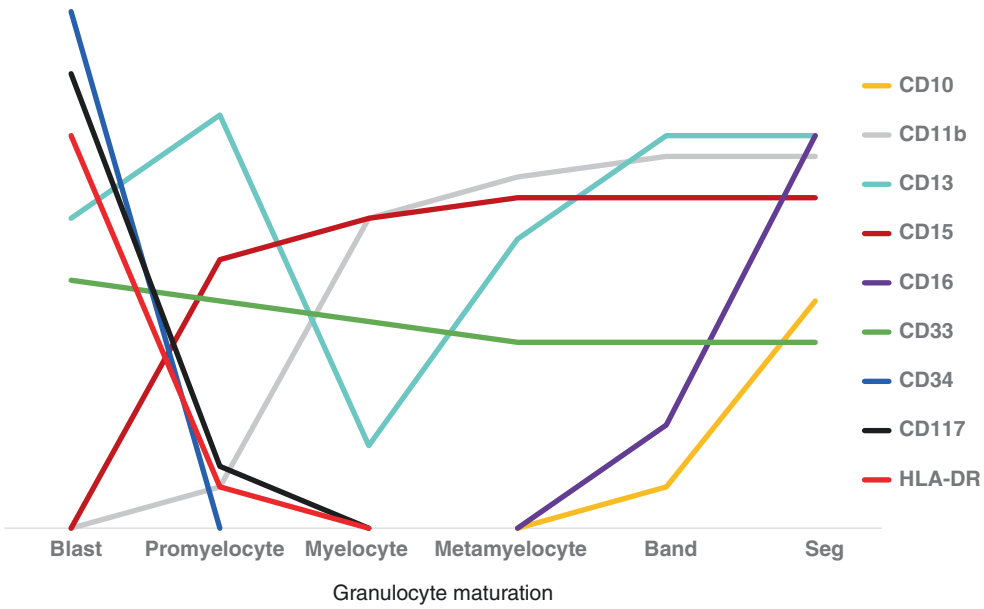

Fig. 22.14 Changes in expression of antigens during myeloid differentiation

Table 22.4 Antigen expression patterns in B cell lymphoproliferative disorders

	CD5	CD10	CD11c	CD19	CD20	CD23	CD25	CD38	CD43	CD103	Cyclin D1	TdT
Chronic lymphocytic leukemia	++	–	–/+	+	+	++	–/+	+/–	+	–	–	–
Mantle cell lymphoma	++	––/+	–/+	++	++	–	–/+	+/–	–/+	–	++	–
Marginal zone lymphoma	–	–	+/–	++	++	––/+	–	–	–/+	–	–	–
Lymphoplasmacytic lymphoma[a]	+/–	–	+/–	++	++	+/–	+/–	+/–	–/+	–	–	–
B cell prolymphocytic leukemia	+/–	–	–	++	++	+/–	–	+/–	+/–	–	–	–
Follicular lymphoma	–	++	–	+	++	–/+	–	+/++	–	–	–	–
Hairy cell leukemia	–	––/+	+++	+++	+++	–	++	–	–	++	–/+	–
B cell lymphoblastic leukemia	–	++	–	++	–/+	–	–	++	+	–	–	++

[a]Most cases have an additional clonal plasma cell population

(b) Classification of B cell lymphoproliferative disorders. B cell disorders are classified in rough accordance with their corresponding maturational stage. Accordingly, antigens that vary with B cell development are useful to help classify these disorders and define the processes.

(i) Antigen expression changes during B cell development are shown in Fig. 22.13.

(ii) A detailed discussion of the phenotyping of B cell malignancies is beyond the scope of this book. Table 22.4 lists the common B cell neoplasms and their typical phenotypic features.

1. CD5. CD5 is expressed normally on T cells and at very low levels on a subset of B cells in the adult, most notably B cells that transition out of the bone marrow into the blood. CD5 is uniformly expressed at moderate to high levels in the B cell lymphoproliferative disorders chronic lymphocytic leukemia (CLL) and mantle cell lymphoma.
2. CD10. CD10 expression varies with B cell development. CD10 is expressed early in B cell development, is lost, and then re-expressed on B cells in the germinal center and lost again. Therefore, B cell neoplasms that arise early in B cell development and during the germinal center stage are CD10 positive. These include:
   (a) B cell lymphoblastic lymphoma/leukemia – progenitor B cell counterpart
   (b) Follicular lymphoma – germinal center B cell counterpart.
   (c) A subset of diffuse large B cell lymphomas arising from the germinal center. Diffuse large B cell lymphomas of germinal center origin have a more favorable prognosis than other diffuse large B cell lymphomas.
   (d) Burkitt lymphoma. Burkitt lymphoma is a highly aggressive lymphoma that arises from germinal center B cells.
3. Absence of CD5 and CD10 characterizes B cells of the marginal zone. Neither antigen is expressed in marginal zone B cell lymphomas.
4. CD23. CD23 is expressed on activated B cells and on a subset of mantle zone B cells. CD23 expression is used to distinguish CLL (positive) from mantle cell lymphoma (negative).
5. Terminal deoxynucleotide transferase (TDT). TDT is expressed during early B cell and T cell development. It is an enzyme that catalyzes the addition of nucleotides to the ends of DNA strands during immunoglobulin heavy chain and T cell receptor gene rearrangements. It is an excellent marker for progenitor B and T cells and is expressed in tumors of early lymphoid development:
   (a) B cell lymphoblastic lymphoma/leukemia
   (b) T cell lymphoblastic lymphoma/leukemia
6. CD38. CD38 is expressed at variable levels throughout B cell maturation. Extremely high levels of expression characterize plasma cells.
   (a) Plasma cell myeloma – Strong expression of CD38 is typical of plasma cell myeloma. The level of expression is similar to that seen on normal plasma cells. A therapeutic monoclonal antibody to CD38, daratumumab, has been developed that is effective in treating plasma cell

myeloma. As a result, identification of plasma cells in patients after daratumumab therapy is not possible using anti-CD38 antibodies. In these situations other strategies are employed to detect plasma cells such as use of antibodies to other markers uniquely expressed or highly expressed on plasma cells such as CD138 or CD317.

(b) CLL – Roughly half of CLL patients have tumor cells that express CD38. These patients have neoplasms that appear biologically immature and resemble the immature B cell stage of development. Mature, post-germinal center B cells normally express little CD38. Studies have shown that patients with CD38-expressing immature-like CLL act more aggressively than those that are CD38 negative.

(c) Detection of abnormal T cells

(i) Identifying neoplastic T cells by flow cytometry is more difficult than detecting malignant B cells for the following reasons:

1. T cell lymphomas and lymphoproliferative processes are uncommon, and most pathologists have less experience with them compared to B cell lymphomas.
2. Clonal assays for T cell neoplasia are not routinely utilized.
3. Multiple normal phenotypic subsets of T cells are present. The phenotypic heterogeneity complicates the recognition of neoplastic T cells. Malignant T cell detection is based primarily on finding aberrant antigen expression which may be masked or mimicked by one or more of the normal T cell subsets.

(ii) Neoplastic T cells can be identified quantitatively via an expanded normal T cell subset or qualitatively through abnormal expression of a T cell antigen. Quantitative changes are not as useful as qualitative abnormalities and can only be used to suggest the presence of a neoplastic process, not document one. Qualitative changes of antigen expression on T cells are the most sensitive and specific means to demonstrate a T cell malignancy.

1. Quantitative methods used in detecting abnormal T cells.

(a) CD4/CD8 ratio. The CD4/CD8 ratio cannot be used to detect clonality of T cells. Unlike light chain expression for B cells, CD4 and CD8 are not expressed as a result of genetic recombination nor controlled by allelic exclusion. As a result, their relative expression in any one individual may be quite variable over time. Although the normal CD4/CD8 ratio ranges approximately from 1 to 3.5:1, the ratio can change rapidly in inflammatory states and infection limiting its usefulness as a marker of neoplasia. Only marked perturbations of the ratio can suggest a neoplastic process. Elevations of the CD4/CD8 ratio greater than 10:1 have been proposed for the diagnosis of Sezary syndrome, a CD4(+) T cell malignancy of skin and blood, for example. However, the criterion is insensitive.

(b) Percentage of CD7-CD4+ T cells. The percentage of CD7-CD4+ T cells has been used to identify neoplastic T cells since CD7 is often absent on CD4 T cell neoplasms. The presence of a CD7-CD4+ T cell population alone is not diagnostic for neoplasia since approximately 15% of normal CD4+ T cells lack CD7. An increase in the percentage of CD7-CD4+ T cells above 40% of the total CD4+ cells has been associated with T cell malignancy, Fig. 22.10. However, similar increases may be observed in reactive conditions impairing the ability to reliably discriminate neoplastic cells from reactive T lymphocytes.

(c) Clonality as assessed by T cell receptor (TCR) variable region beta (Vbeta) expression. This analysis is expensive to perform and not routine. Mature T cells express TCRs composed of one alpha chain and one beta chain unique to each cell similar to immunoglobulin expression on B cells. Like B cells, any one T cell only expresses a single TCR. The beta chain of the TCR is produced from one of 30 variable

region gene segments. Therefore, a population of polyclonal T cells will contain cells with an equal distribution of the TCR Vbeta gene products. Clonality can be determined by demonstrating abnormal expansion of a population of T cells carrying only one of the 30 TCR Vbeta gene segments, Fig. 22.15. The method is essentially like a 30-part kappa/lambda ratio. Limitations include:

(i) Commercially available antibodies react with only 70% the possible TCR Vbeta region gene products. Thirty percent of suspected neoplasms with not react.

(ii) There is poor sensitivity when the neoplastic population represents only a small fraction of cells.

(iii) Many antibodies are required for an analysis making it costly and necessitating staining of a large number of cells in multiple tubes. Therefore, TCR Vbeta analysis is rarely used.

2. Qualitative methods for identifying T cell neoplasia. For the reasons given above, analyses for determining T cell neoplasia have focused on antigen expression abnormalities.

(a) Abnormalities in the expression level of T cell-associated antigens CD2, CD3, CD4, CD5, CD7, and CD8 have all been described. Abnormal CD3 expression is most frequently encountered, usually atypical low-level expression, occurring in roughly 70% of T cell neoplasms, Fig. 22.10. Ninety percent or more of T cell malignancies will demonstrate atypical phenotypic expression of one or more of these antigens. Care must be taken to ensure that altered antigen expression is not related to a normal T cell subset or induced by a reactive process.

(b) CD10. CD10 is expressed on germinal center T lymphocytes and precursor T cells. Expression of CD10 on T lymphocytes recovered from blood or bone marrow, sites normally lacking these cells, is a strong indicator of a neoplastic process.

(c) Aberrant myeloid antigen expression. Aberrant expression of myeloid-associated antigens on T cells is a rare but useful finding in T cell neoplasia. CD13 may be expressed on anaplastic T cell lymphoma cells, for example.

(d) Classification of T cell lymphoproliferative disorders. T cell neoplasm classification is less cor-

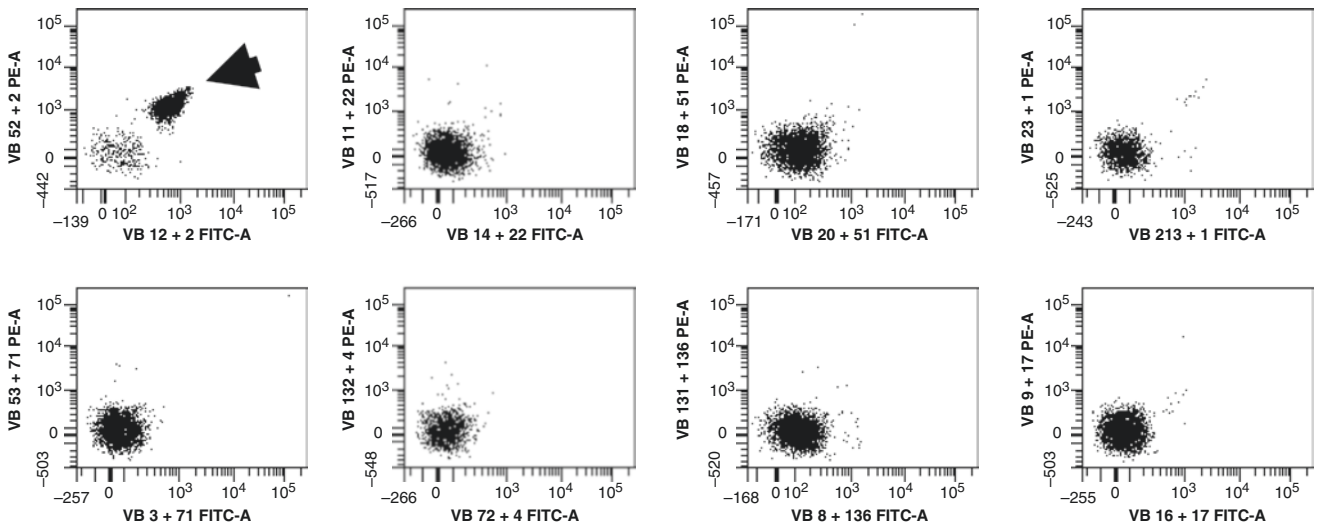

Fig. 22.15 Clonality of T cells determined by TCR Vbeta expression. Virtually all the T cells in the example demonstrate expression of a single TCR Vbeta type (arrow) indicative of clonality

Table 22.5 Antigen expression in T cell lymphoproliferative disorders

	CD2	CD3	CD4	CD5	CD7	CD8	CD10	CD25	CD26	CD56	αβ/γδ	TdT
Mycosis fungoides/Sezary syndrome	++	+	+/++	++	−−/+	−	−−/+	−/+	−−/+	−	αβ	−
T cell prolymphocytic leukemia	++	++	++	++	++	−	−	−	++	−	αβ	−
HTLV-1 associated leukemia/lymphoma	++	+	++	++	−−/+	−	−	++	−−/+	−	αβ	−
Large granular lymphocytic leukemia	++	+/++	−	−−/+	−/+	++	−	−/+	+	−/+	αβ	−
Hepatosplenic T cell lymphoma	++	+	−	−/+	+	−−/+	−	−	?	+/−	γδ	−
Angioimmunoblastic T cell lymphoma	++	+	++	+	−−/+	−	+	−/+	−/+	−	αβ	−
T cell lymphoblastic leukemia	+/−	−−/+	+/−	+/−	++	+/−	−/+	−	+	−	−−/αβ	+

? = unknown

related with T cell development than B cell disorders but is nonetheless dependent on phenotype. A summary table of typical phenotype of T cell malignancies involving blood and bone marrow is shown, Table 22.5.

(e) Lineage assignment for acute leukemia. The therapy for acute leukemia is dependent of the lineage of the cells involved. It is critical to correctly define the leukemic process as either a B, T, or myeloid neoplasm, Fig. 22.16. Theoretically this would appear simple since the expression of many antigens is known to be restricted to specific cell lineages. However, in practice, leukemic cells often display phenotypic aberrancies and express antigens normally associated with other cell lineages. Therefore, criterion has been developed to help in lineage assignment, Table 22.6. In unusual circumstances a leukemia will demonstrate a mixed phenotype pattern demonstrating differentiation toward more than one lineage, most commonly B cell and myeloid.

(f) Assessment of abnormal myeloid cells and maturation.

(a) Enumeration of myeloid lineage assignment for blasts. Flow cytometry is particularly useful for the enumeration of myeloid blasts in acute myeloid leukemia and preleukemic (myelodysplastic) states, Fig. 22.11. Acute myeloid leukemia is defined as the presence of 20% or more blasts in the peripheral blood or bone marrow. Myeloid neoplasms with blast percentages below these levels are classified as myelodysplastic syndromes. Cells expressing CD34 and CD117 (c-kit) are known to be primitive stem cells (blasts) committed to the myeloid lineage. These cells normally represent ~0.5% of normal bone marrow constituents and are extremely rare in peripheral blood constituting roughly ~0.01%. An increase in CD34+CD117+ cells is characteristic of myelodysplastic syndromes and may indicate an acute myeloid leukemia.

(b) Classification of acute myeloid leukemia. Acute myeloid leukemia is classified based on a combination of cytogenetic, molecular, and phenotypic findings. The phenotype of the cells clarifies the lineage for classification and is useful to predict cytogenetic abnormalities.

(i) Acute promyelocytic leukemia (APL) is an acute myeloid leukemia characterized by blasts that lack CD34 and HLA-DR, Fig. 22.16.

(ii) Acute myeloid leukemia with t(8:21) is associated with many mature granulocytes and myeloid blasts that typically express CD19 and CD56.

(iii) Acute myeloid leukemias with monocytic differentiation (M4 or M5) contain blasts with a monocytic phenotype typically expressing, at least in part, monocyte markers CD14, CD11c, and CD64.

(c) Atypical expression pattern of CD11b, CD16, and CD13. The normal structured maturational pattern of expression of these antigens may be altered in myeloid neoplasia, particularly myelodysplastic syndromes. However, similar alterations may occur in inflammatory states. Therefore, care needs to be taken in not overinterpreting changes that are observed.

(d) Aberrant expression of lymphoid antigens. Abnormal expression of lymphoid-associated antigens CD2, CD5, CD7, or CD56 may occur on CD34+CD117+ blasts in neoplastic conditions even if the cells are not increased. This is a strong indicator of a neoplasm, Fig. 22.17.

(e) Atypical loss of antigen expression on myeloid blasts. Loss of an antigen normally expressed on myeloid stem cells also strongly suggests neoplasia. HLA-DR, CD117, or CD34 may aberrantly be lost on these cells.

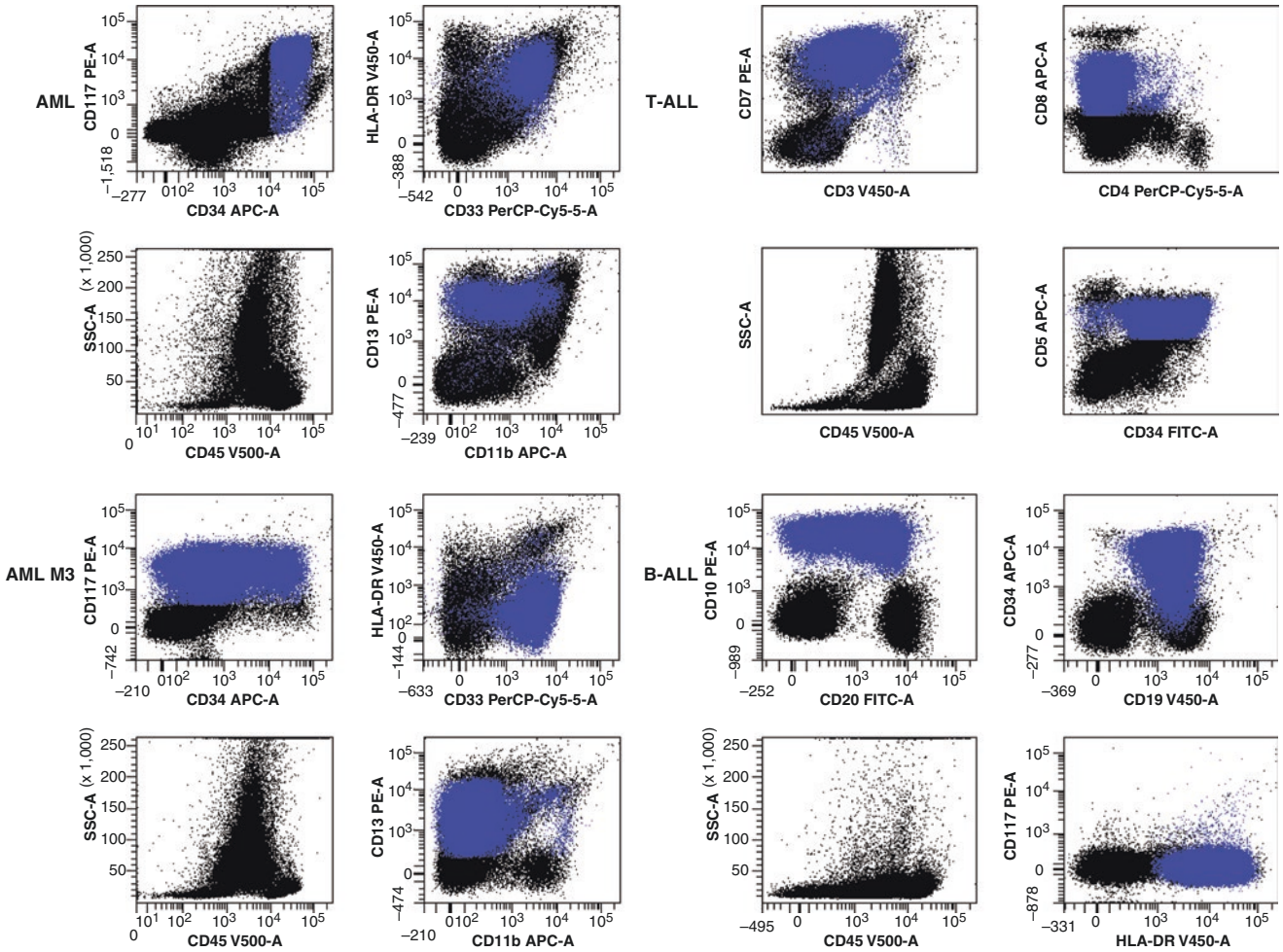

Fig. 22.16 Flow cytometry of acute leukemia. Representative dot plots are shown for acute myeloid leukemia (AML), T cell lymphoblastic leukemia/lymphoma (T-ALL), acute myeloid leukemia, M3 type (AML M3, acute promyelocytic leukemia), and B cell lymphoblastic leukemia/lymphoma (B-ALL)

Table 22.6 Lineage assignment in acute leukemia

Myeloid lineage
Myeloperoxidase
Monocytic differentiation (CD14, CD11c, CD64, lysozyme, non-specific enolase)
T cell lineage
Strong cytoplasmic expression of CD3
Surface CD3 expression
B cell lineage
Strong CD19 expression and strong expression of either CD79a, CD22, or CD10
Weak CD19 expression and strong expression of two other antigens, CD79a, CD22, or CD10

(f) Atypical expression of CD56 on mature myeloid elements. CD56, an antigen normally associated with natural killer cells, is often expressed on mature monocytes and granulocytes in clonal myeloid processes such as myelodysplastic syndromes and myeloproliferative neoplasms. The atypical expression is unexplained but may be seen in up to 50% of these disorders. Low-level CD56 expression may also occur after therapy with growth-stimulating factors requiring careful correlation with clinical history when observed.

(g) Loss of antigen expression on mature myeloid cells. Mature myeloid cells may demonstrate atypical loss of antigen expression. Examples include loss of CD14 or CD13 on monocytes and decreased or absent CD10 expression on segmented neutrophils. These changes are less common than aberrant CD56 expression.

(g) Detection of neoplastic plasma cells. Plasma cells are notoriously poorly recovered by flow cytometry. This may require collecting

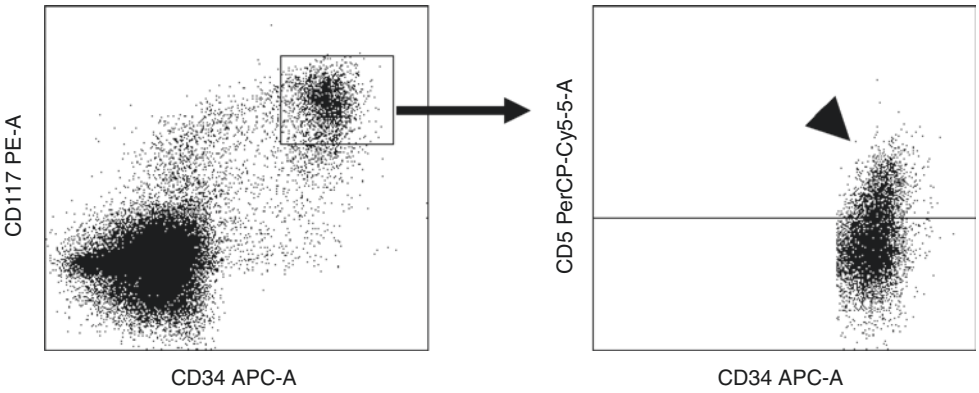

Fig. 22.17 Atypical expression of CD5 on myeloblasts (CD34+CD117+ cells). Myeloblasts demonstrate low-level partial expression of CD5, a finding not observed on normal myeloblasts indicating a neoplastic process. Control cutoff level is shown as horizontal line

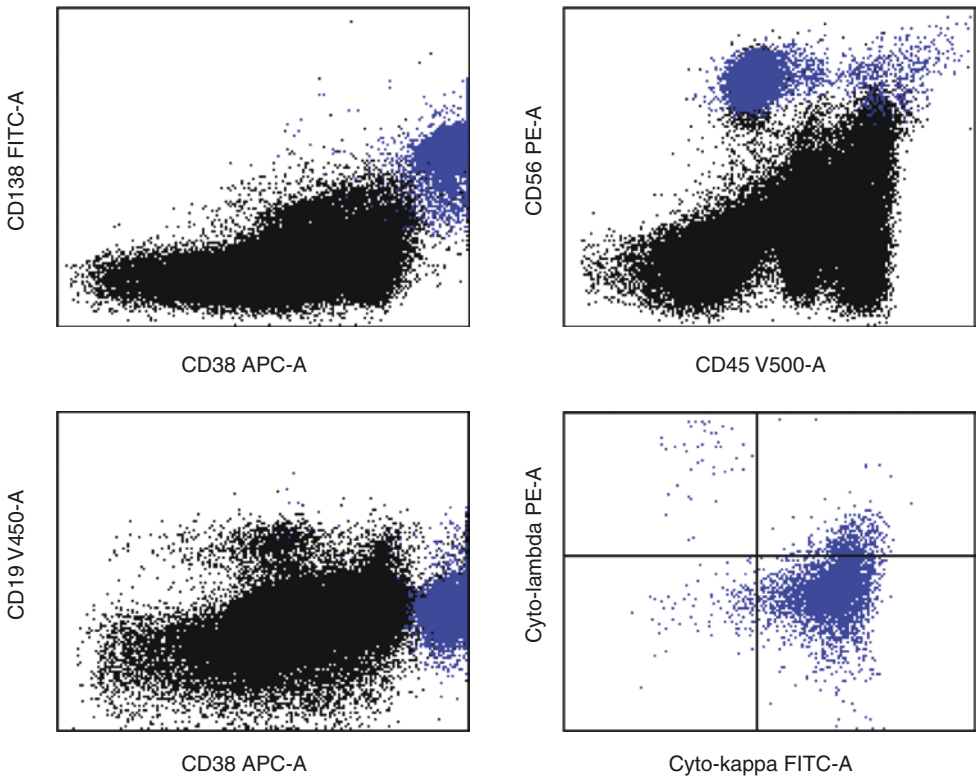

Fig. 22.18 Flow cytometry of plasma cell myeloma. Representative dot plots are shown

more cells than normal (500,000 or more) than is typically procured for a clinical specimen. Regardless, neoplastic plasma cells have unique features that can be used to distinguish them from normal plasma cells, Fig. 22.18. Subtle features distinguish neoplastic plasma cells in plasma cell myeloma compared to those that may accompany a lymphoma such as lymphoplasmacytic lymphoma.

(a) Plasma cells, both reactive and neoplastic, demonstrate very strong expression of CD38 and express CD138. These two antigens in combination are very sensitive for identifying plasma cells of any type.

(b) Phenotypic alterations of plasma cells in plasma cell myeloma.

(i) CD56 and CD117. Plasma cell myeloma plasma cells commonly demonstrate atypically expression of CD56 (~70%) and CD117 (~50%).

(ii) Absence of CD19. Myeloma plasma cells almost always lack CD19, normally expressed at low to moderate levels on reactive plasma cells and clonal plasma cells associated with lymphoma.

(iii) Decreased or absent expression of CD45. Atypical low to absent expression of CD45 is a common finding in

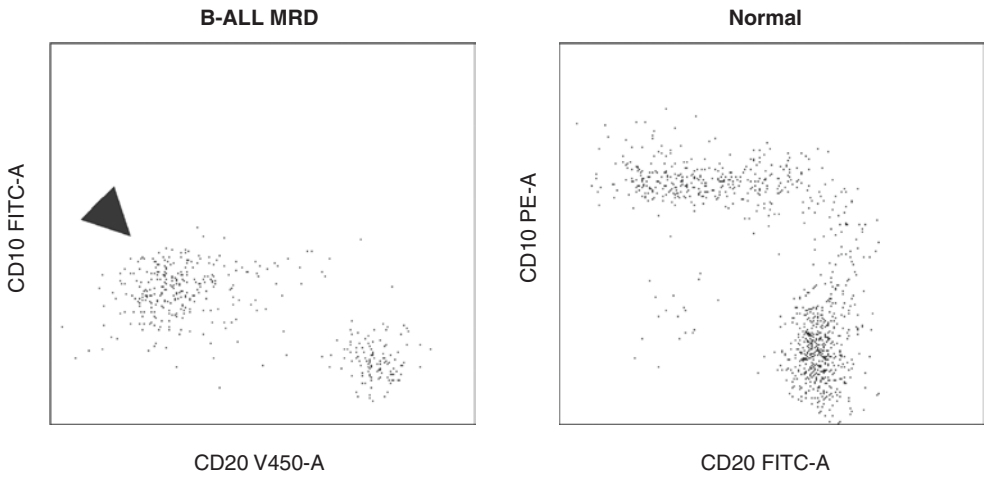

Fig. 22.19 Minimal residual disease in B cell lymphoblastic leukemia/lymphoma. Abnormal low-level expression of CD10 is seen (left) compared to the normal expected pattern (right). The atypical antigen expression pattern is indicative of neoplastic cells

plasma cell myeloma cells. Additional antigens abnormally expressed on myeloma cells include CD27 and CD81.

(iv) Phenotypic aberrancy as described above is observed in close to 100% of plasma cell myelomas.

(c) Clonality. Clonality of plasma cells can be documented by use of intracellular staining for light chains. Oftentimes this is not necessary due to the common phenotypic abnormalities observed in myeloma cells.

(d) Even though recovery of plasma cells by flow cytometry is relatively poor, the detection of any phenotypically abnormal plasma cell population after therapy for plasma cell myeloma has been shown to be predictive for recurrence.

(h) Minimal residual disease detection. The morphologic detection limit for neoplastic cells is roughly 2–5%. The presence of detectable disease below which can be visually identified is referred to as *minimal residual disease (MRD)*. Additional techniques are required for this degree of sensitivity. Flow cytometry can identify neoplastic cells from acute leukemia and other disorders down to 0.01% making it an excellent method to monitor MRD. Studies have shown that detection of low-level disease after therapy is an important predictor of disease recurrence in a wide range of hematologic malignancies. Therefore, assessment of MRD is commonly performed in patients undergoing therapy for hematopoietic neoplasms.

(a) Both flow cytometry and molecular methods afford the ability to detect neoplastic cells down to very low levels. Currently flow cytometry is method of choice for MRD detection for B and T cell lymphoblastic lymphoma/leukemia, chronic lymphocytic leukemia, hairy cell leukemia, and plasma cell myeloma and is complimentary with molecular methods for the detection of MRD in individuals with acute myeloid leukemia.

(b) Method. Two main principles are used for the detection of MRD by flow cytometry.

(i) The identification of a cluster of cells that lie outside the normal expected maturational pathway. This is the main method used to detect MRD in B cell lymphoblastic leukemia/lymphoma, Fig. 22.19.

(ii) The identification of a unique neoplastic cell-associated phenotype, often referred to as *a leukemia-associated phenotype*. Examples are CD5 expression on myeloid blasts and the expression of CD56 on plasma cells.

A cluster of 20 or more cells satisfying the criteria is generally regarding as sufficient evidence of MRD. Therefore, a minimum of 200,000 total events must be collected to establish MRD down to 0.01%. In most instances many more cells are collected and evaluated to ensure this level of sensitivity if attained.

Summary

Flow cytometry is a method of cell analysis used in the evaluation of hematologic and immunologic disorders. This chapter reviews the basic operational principles of flow cytometry and its use as a diagnostic tool. Applications and uses in the evaluation of benign hematology, platelet disorders, immunology, transplantation, and neoplastic hematology are reviewed. The chapter should give the student a fundamental understanding of flow cytometry and its utility in hematology.

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Web Resources

Flow Cytometry Overview

https://www.bdbiosciences.com/us/support/training/s/itf_launch
http://www.beckman.com/coulter-flow-cytometry/practical

Genetics in Hematologic Disorders: Implications of Recurring Chromosome Abnormalities and Gene Mutations

23

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Introduction

Hematologic malignancies are characterized by numerous chromosome abnormalities, including translocations and inversions, gains and losses, deletions and duplications, and various genomic imbalances and gene mutations. Conventional chromosome analysis, fluorescence in situ hybridization (FISH) test, genomic microarray, and next-generation sequencing (NGS) are the specific techniques used to detect disease-related chromosome aberrations, genomic imbalances, and gene mutations. Many chromosome abnormalities are closely associated, and sometimes uniquely, with morphologically and clinically distinct subtypes of leukemia and lymphoma, and are one of the most reliable predictors of disease prognosis. The detection of these chromosome abnormalities assists not only in establishing diagnosis but also provides important prognostic information and risk stratification and helps decide efficient treatment based on genetic targets, as well as monitor treatment response and disease progression [1, 2].

Techniques

Chromosome abnormalities and gene mutations may be detected by conventional chromosome analysis, FISH, genomic microarray, and NGS techniques. Each technique has special requirements in experiments and various advantages and limitations of quality and applications (Table 23.1).

Table 23.1 Comparison of cytogenetics, FISH, microarray, and next-generation sequencing techniques

Techniques	Cytogenetics	FISH	Microarray	NGS
Resolution	+	++	+++	++++
Sensitivity	+	+++	++	++
Copy neutral LOH	–	–	+++	+/–
Cell division	+	–	–	–
Balanced translocations/ inversions	+	+	–	+
Multiple clones	+	–/+	–	+/–
Screen for novel lesions	+	–	+	+

Chromosomal Analysis (Karyotyping)

Chromosomal analysis requires fresh and viable cells from bone marrow aspirate, peripheral blood, lymph nodes, or tumor tissues. It starts with short-term cultures, followed by arresting cells at the metaphase phase of the cell cycle, hypotonic treatment and fixation, dropping and banding metaphase cells, and careful analysis for numerical and structural chromosome abnormalities. Acute leukemia cells often grow actively and divide spontaneously in short-term culturing. For low-grade B- or T-cell leukemia or lymphoma, specific mitogens, such as PHA or pokeweed, and IL-2/CpG oligonucleotide may be used in promoting cells to grow and divide in cell culturing. *Advantages:* (1) Whole genome level analysis to check all chromosome abnormalities, which may identify novel chromosome aberrations. (2) Precise analysis at single cell level to reveal clonal evolution and heterogenicity. (3) Chromosome abnormalities observed may reflect tumor cell proliferation and biology. *Limitations:* (1) Analysis at low-level chromosome band resolution. (2) The analysis success heavily relies upon cell division and thus is not feasible in specimens with non-dividing, or fixed cells. (3) It requires extensive skills and knowledge in analysis and high chromosome preparation quality.

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Fluorescence In Situ Hybridization (FISH) Testing

FISH test uses specific DNA probes to detect various chromosome and gene aberrations, including numerical and structural chromosome abnormalities, such as translocation, inversion, deletion and gain, amplification, etc., in a large number of metaphase and interphase cells as well as tissue sections. In comparison with chromosome analysis, FISH technique has a high sensitivity, specificity, and resolution.

Two main types of FISH probes are routinely used in clinical services. (1) Centromere-specific probes can reveal chromosome gain or loss, such as −7 in AML and MDS and +12 in CLL. (2) Locus-specific probes, such as dual fusion probes for *BCR* and *ABL1* to detect t(9;22) in CML and B-cell ALL or *MLL* (now called *KMT2A*) break-apart probes for various 11q23 translocations in leukemia, such as t(4;11) in B-ALL and t(9;11) in AML. A dual fusion set with two probes for both involved genes allows a precise detection of specific chromosome translocations or inversion, whereas a break-apart probe set with two probes that cover the 5′ and 3′ regions of the relevant gene locus, respectively, shows all translocations with a breakpoint region franking by the probes but cannot determine partner chromosome or genes involved.

Advantages: (1) It is relatively straightforward in procedure and analysis and generally has a quick turnaround time of 1–2 days. (2) FISH can be applied to both dividing (metaphase) and nondividing (interphase) cells; therefore it is suitable in diseases with low mitotic index, such as CLL, myeloma, and in tissue preparations, cytospin prep, peripheral blood smear, touch prep, and formalin-fixed and paraffin-embedded (FFPE) tissue section. (3) FISH analysis is at single-cell level and thus may be combined with immunophenotyping or morphological assessment and allows simultaneous visualization of abnormal FISH signal patterns and cell lineage features, which is particularly helpful in patients with coexistence of lymphoid and myeloid neoplasm, such as CLL and MDS in elderly patients. (4) FISH may be applied to enriched or sorted cell population by flow cytometry techniques, such as multiple myeloma, various lymphomas, or myeloid populations. This significantly increases the sensitivity of FISH tests, allowing determining minimal residual disease status precisely, as well as in lymph nodes or other FFPE tissues with scattered tumor cells. *Limitations:* (1) It applies only to the targeted regions and genes where probes bind to, and thus, it is a targeted test, not whole genome analysis. (2) Due to relatively large probe sizes, FISH may not detect small deletions or inversion within the franking regions.

Genomic Microarray Testing

Advantages: Microarray technique employs thousand to million probes over the entire genome to detect genomic imbalances, such as gain or loss of relevant chromosome regions in tumor cells. Genomic microarray with single nucleotide polymorphism (SNP) probes also reveals copy-neutral loss of heterozygosity (CN-LOH). Microarray tests are particularly useful in revealing whole genomic abnormalities and CN-LOH features in solid tumors in FFPE preparations or in those without dividing cells. The analysis also defines a precise size of deletion or gain region and genes involved. *Limitations:* (1) Balanced chromosome translocations, inversions, or insertions cannot be detected. (2) The sensitivity of the test is approximately only 10–20%; therefore, it cannot reproducibly detect small clones below 20% of tumor content in a specimen.

Next-Generation Sequencing (NGS) Techniques

NGS analysis of targeted gene fusions and mutations rapidly becomes available for routine clinical diagnostic service of leukemia and lymphoma and allows better risk stratification and selection of targeted therapies. *Advantages*: (1) It may precisely detect recurring leukemia-specific gene fusion transcripts or (2) identify novel gene fusions that define specific leukemia entities, such as BCR-ABL1-like B-cell acute lymphoblastic leukemia. (3) It has higher sensitivity and is more comprehensive than single-cell mutations tests by traditional Sanger sequencing and thus may apply for detecting minimal residual disease and the analysis of multiple gene mutations in a panel reaction. *Limitations*: (1) It requires specific and expensive sequencing equipment; (2) data analysis and interpretation heavily depend on bioinformatic pipelines and experience; (3) copy number gain or loss cannot be definitely detected, partly depending on tumor content, such as 20% or above.

Selection of Karyotyping, FISH, and/or Microarray Tests in Clinical Diagnostics

Selection of one or more of these tests above depends on disease types, disease status, specific targets, and history of previous analysis results. The goal is to appropriately use one or more tests to maximally detect and clarify chromosome abnormalities that are critical for diagnosis, prognosis, and monitoring treatment responses and, at the same time, to avoid redundant or non-informative follow-up tests and cost.

Chromosome analysis should be performed as initial screening for chromosome abnormalities in all newly diagnosed or suspected acute and chronic myeloid neoplasia and in acute lymphoblastic leukemia. FISH tests at the front line are helpful in certain diseases with specific diagnostic or prognostic targets, such as FISH tests for t(9;22) in CML and B-ALL and for t(15;17) in APL [3]. FISH is critical in leukemia with recurring cryptic chromosome abnormalities, such as *CHIC2* deletion/*PDGFRA* in hematological neoplasia with eosinophilia and t(12;21) *ETV6/RUNX1* fusion in B-ALL. FISH may be necessary to clarify specific chromosome abnormalities in samples with suboptimal chromosome quality and incomplete chromosome analysis. For lymphoma patients, FISH for specific targets would be more helpful than chromosome analysis in determining a precise diagnosis and prognosis, such as t(11;14) in suspected MCL, deletion of 11q (*ATM*) and 17p(*TP53*) in CLL panel, as well as *MYC* translocations in high-grade B-cell lymphomas [4].

For follow-up samples with residual disease or in remission, FISH for the known chromosome abnormalities from a diagnostic specimen is more sensitive and objective than chromosome analysis in determining the clone size. FISH is also very precise in determining engraftment status in patients who received sex-mismatched allogeneic hematopoietic cell transplantation. Chromosome analysis should be repeated in all samples with relapse or in progression in order to analyze all chromosomes for potential clonal evolution and novel chromosome abnormalities.

Microarray tests will provide a high resolution of whole genomic level detection and therefore is particularly useful for those diseases with recurring chromosome gain or loss, such as MDS, CLL, and myeloma, and may replace multiple FISH tests in these diseases. However, FISH may be critical to detect an early small clone of certain targets that are significant in prognosis, such as *TP53* deletion in myeloma and CLL. In addition, microarray test cannot detect chromosome translocations and inversions that are detectable by chromosome analysis, FISH or reverse PCR, or NGS tests.

Significance of Detection of Cytogenetic Abnormalities

The detection of specific chromosome translocations and gene fusions helps establish a precise diagnosis of leukemia and lymphoma, and the detection of recurring gene mutations in hematological neoplasia provides important prognostic information and guides the selection of targeted genetic-based therapies [1, 3, 4, 5].

(a) Establish a specific diagnosis, such as t(9;22) in CML, t(15;17) in APL, and t(11;14) in mantle cell lymphoma.
(b) Determine disease prognosis, i.e., t(8;21), inv(16)/t(16;16) and t(15;17), and t(12;21), and hyperdiploid clones are associated with favorable prognosis AML and B-cell ALL, respectively, whereas complex karyotype, particularly with loss of chromosome 17 or deletion of *TP53*, is indicative of poor prognosis in AML, and t(9;22) is a strong predictor of high risk in B-ALL.
(c) Monitor treatment response and disease progression when new chromosome abnormalities or clonal heterogeneity and subclones occur. FISH analysis provides a quick and precise engraftment status in patients after sex-mismatched stem cell transplant.
(d) Help select appropriate treatment options based on genetic abnormalities. Certain chromosomal changes may predict for response (or nonresponse) to specific therapies, i.e., tyrosine kinase inhibitors, such as imatinib in CML with t(9;22) and early treatment with ATAR for patients with APL and t(15;17).
(e) Understand the pathogenesis of the disease and identify genes that are involved in leukemogenesis.

Understanding the Cytogenetic Report

Definition of Clonality According to the International System for Human Cytogenetic Nomenclature (ISCN), [6] an abnormal clone is established with (1) two or more cells with the same structural rearrangement, such as translocations, deletions, or inversions or with gain of the same chromosome or (2) three cells with loss of the same chromosome. However, a single cell with a known abnormality from previous samples, or confirmed by a second test, such as FISH, also confirms the presence of an abnormal clone.

Commonly Used Cytogenetic Terms and Definition (ISCN) A karyotype is described first by the total number of chromosomes and the sex chromosome component, followed by abnormalities of autosomal chromosomes listed in numerical order irrespective of aberration type. Chromosome abnormalities are written in order of the involved chromosomes in the first set of parentheses and the breakpoints in a second set, followed by the total number of the analyzed cells with the clone. A typical karyotype in a male patient with CML is written as 46,XY,t(9;22)(q34.1;q11.2)[20]. The most commonly used terms in conventional chromosome studies are listed in Table 23.2 with explanation and examples of abnormalities.

In addition, the interpretation of chromosome abnormalities and their correlation with morphology and association with prognosis is important. Complex karyotype and monosomal karyotypes are associated with unfavorable

Table 23.2 Common cytogenetic and FISH karyotype terms and definitions according to the International System of Human Cytogenetic Nomenclature (2016) [6]

Terms/ symbols	Definitions	Examples
+ or −	Gain or loss of chromosomes	+12, −7
add	Addition of unknown material	add(5)(q31)
del	Deletion of chromosome material	del(13)(q12q14)
der	Derivative chromosome of structural abnormalities	Der(7)add(7)(p11.2)del(7)(q11.2q32)
dic	Dicentric chromosome	Dic(5;17)(q11.2;p11.2)
dup	Duplication of chromosome bands	dup(1)(q12q41)
idem	Identical to the stem clone	46,XY,t(9;22)(q34;q11.2)[2]/47,idem,+8[18]
i	Isochromosome	i(17)(q10)
inv	Inversion within chromosome arm or both arms	Inv(16)(p13.1q22), inv(3)(q21q26.2)
ins	Insertion	Ins(12;4)(q13;q21q31)
mar	Marker chromosome	mar1, mar2
r	Ring chromosome	r(7)(p11.2q22)
t	Translocation of two or more chromosomes	t(9;22), t(11;14)
cp	Composite karyotype of multiple various aberrations (not all cells have all abnormalities)	47~49,XY,+8,+11,+18[cp5]
nuc ish	Interphase FISH test	Nuc ish (ABL1,BCR)x3 (ABL1 con BCRx2)[190/200] i.e., t(9;22) in CML
sep	Separation of break-apart probes	(MLLx2)(5′MLL sep 3′MLLx1)[40/200] i.e., 11q23/MLL translocation positive
con	Signal fusion	See above

prognosis in AML and MDS, more often in therapy-related myeloid neoplasia. The definition of complex karyotype refers to clones with three or more independent chromosome abnormalities; these complex clones often carry marker chromosomes with unknown origin of the centromere, ring chromosome, and derivative chromosome with complex abnormalities. A monosomal karyotype is defined as loss of at least two autosomal chromosomes or loss of one autosomal chromosome and one or more structural chromosomal abnormalities, excluding ring and marker chromosomes.

The chapter below describes the most common chromosome abnormalities and gene mutations in various hematological diseases and discusses their significance in disease diagnosis and biology and impact in assessing prognosis and therapeutic choices.

Table 23.3 Recurring chromosome abnormalities that define unique chronic myeloid neoplasia

Chromosome abnormalities	Genes involved	MPN
t(9;22)(q34;q11.2)	BCR/ABL1	CML
t(5;12)(q33;p13)	PDGFRB/ ETV6	CMML
−7	?	JMML
del(4q12)/t(4q)	FIP1L1/ PDGFRA	Myeloid and lymphoid neoplasia with eosinophilia
t(5q32), multiple	PDGFRB	
t(8p11.2), multiple	FGFR1	
t(8;9)(p22;p24.1)	PCM1/JAK2	

CML chronic myeloid leukemia, *CMML* chronic myelomonocytic leukemia, *JMML* juvenile myelomonocytic leukemia

Chronic Myeloid Leukemia (CML) and t(9;22)/ Philadelphia Chromosome

CML is the best model for our understanding of the mechanisms of genetic abnormalities in leukemogenesis. CML was the first cancer to be associated with a recurring chromosome abnormality, i.e., Philadelphia chromosome in 1960 [7], and later defined as t(9;22) in 1973 [8]. CML was also the first disease that was found to result in a novel *BCR/ABL1* fusion gene at molecular level in the early 1980s [9]. Furthermore, CML serves as the first cancer with an efficient genetic-based treatment that directly targets the genes responsible for the pathogenesis of the disease, i.e., imatinib in1996 [10].

The t(9;22) is the hallmark of CML, and results in the *BCR/ABL1* fusions, and is detectable by chromosome analysis in >90% of CML patients (Table 23.3) [5]. In the remaining 2–10% of patients with CML, a variant of the t(9;22), including three-way translocations, is observed. In some rare cases, the insertion of the *BCR* gene into the *ABL1* gene or vice versa occurs and can be detected only by FISH or molecular tests. The *BCR/ABL1* fusion is a constitutively active tyrosine kinase, which is implicated in the origin of CML and is the primary genetic target for tyrosine kinase inhibitor, such as imatinib.

When CML progresses, additional chromosome abnormalities, including gain of Ph chromosome, +8, i(17q), and +19, are most common. The presence of these additional chromosome abnormalities is considered CML in acceleration. The t(3;21) and inv(3q)/t(3;3) are also frequently observed in CML in progression that results in the overexpression of the EVI1 gene (3q26.2). With imatinib and other TK inhibitors as the frontline drugs, the outcome of CML has been significantly improved. Chromosome analysis, FISH tests, and qualitative PCR for monitoring the t(9;22) and BCR/ABL1 transcript level remain critical in CML.

Table 23.4 Recurring chromosome abnormalities defining unique AML according to WHO 2016 classification and their prognostic significance

Chromosome abnormalities	Genes involved	Clinical prognosis
t(8;21)(q22;q22)	RUNX1 (AML1)/ RUNX1T1 (ETO)	Favorable
inv(16)(p13.1q22)/t(16;16)(p13.1;q22)	CBFB/MYH11	Favorable
t(15;17)(q24;q21)	PML/RARA	Favorable
t(9;11)(p22;q23)	KMT2A (MLL)/ MLLT3 (AF9)	Intermediate
t(6;9)(p23;q34)	DEK/NUP214	Poor
inv(3)(q21q26.2)/t(3;3)(q21;q26.2)	GATA2, MECOM	Poor
t(1;22)(p13;q13)	RBM15/MKL1	Poor
t(9;22)(q34;q11.2)	BCR/ABL1	Poor

Table 23.5 Prognostic significance of recurring chromosome abnormalities in AML according to Grimwade et al. [11]

Favorable (10%)
t(15;17)
t(8;21)
inv(16)/t(16;16)
Intermediate (70%)
Normal karyotype (40%)
t(9;11)
Other entities not as favorable or adverse
Adverse (20%)
Inv(3)/t(3;3), other 3q abnormalities (excluding t(3;5)),
add(5q)/del(5q)/−5,
add(7q)/del(7q)/−7,
add(17p)/del(17p)/−17,
11q23 (MLL) translocations, including t(6;11), t(10;11),
t(9;22),
Complex karyotype (three or more unrelated chromosome abnormalities)

Acute Myeloid Leukemia (AML)

With combination of morphology, immunophenotype, genetics, and clinical features, many subtypes of acute and chronic leukemias are classified in the WHO classification system (Table 23.4) [3]. Chromosomal abnormalities are detectable in 50–60% of de novo AML and up to 92% of therapy-related myeloid neoplasia. t(15;17), t(8;21), inv(16)/t(16;16), and 11q23/*MLL*(*KMT2A*) translocations are unique chromosome translocations and inversions in AML, whereas deletion or loss of chromosomes 5, 7, 17, +8, del(20q), del(9q), and del(12p) are the most common recurring chromosomal deletions and gains. In addition, t(6;9), inv(3)/t(3;3), t(1;22), and t(9;22) are also included in the WHO classification system as AML with recurrent genetic abnormalities. Detection of t(8;21), inv(16)/t(16;16), and t(15;17) establishes a diagnosis of AML with these unique chromosome abnormalities, regardless the blast cell percentage in the bone marrow or peripheral blood specimens. These balanced translocations or inversions often result in a chimeric fusion gene encoding a novel protein that participates in leukemogenesis of AML and are associated with various prognoses (Table 23.5) [11].

CBF Leukemia with t(8;21)or inv(16)/t(16;16)

The core-binding factor (CBF) leukemias are defined as AML with either t(8;21) or inv(16)/t(16;16) that involves the core-binding factor (CBF) unit A (*RUNX1/AML1*) and unit B (*CBFB*), respectively. The core-binding transcription factor (CBF) complex binds directly to an enhancer core motif at the transcriptional regulatory regions of several genes, including IL3, GM-CSF, the CSF1 receptor, myeloperoxidase, and neutrophil elastase. These genes are critical to hematopoietic stem and progenitor cell growth, differentiation, and function.

The t(8;21) is one of the most common chromosome translocations in children and adult patient with AML. This translocation results in a chimeric fusion of the *RUNX1* (also called *AML1*) gene (21q22) with the *RUNX1T1 (ETO)* gene (8q22) (Fig. 23.1). The RUNX1 protein is one of the transcription factor family members with homology to the pair-rule Drosophila gene runt.

inv(16) or t(16;16) is another recurring chromosome abnormality in AML often associated with monocytic and granulocytic differentiation and with abnormal eosinophils in the bone marrow. The inversion results in a novel chimeric fusion between the *CBFB* gene at 16q22, and the *MYH11* gene at 16p13.1 (Fig. 23.2). Both novel fusion proteins resulting from t(8;21) or inv(16)/t(16;16) disrupt the normal function of the CBF complex and result in the repression of transcription.

CBF leukemia is generally associated with a favorable prognosis with high remission and long-term survival. In AML with t(8;21), loss of a sex chromosome (−X or −Y) or a del(9q) is frequently observed as secondary chromosome abnormality with no significant impact on the favorable prognosis. In AML with inv(16)/t(16;16), gain of chromosome 22 is frequent and may be associated with a more favorable prognosis than those with inv(16)/t(16;16) only. However, C-KIT mutations are not rare in CBF AML and may lead to less favorable outcome.

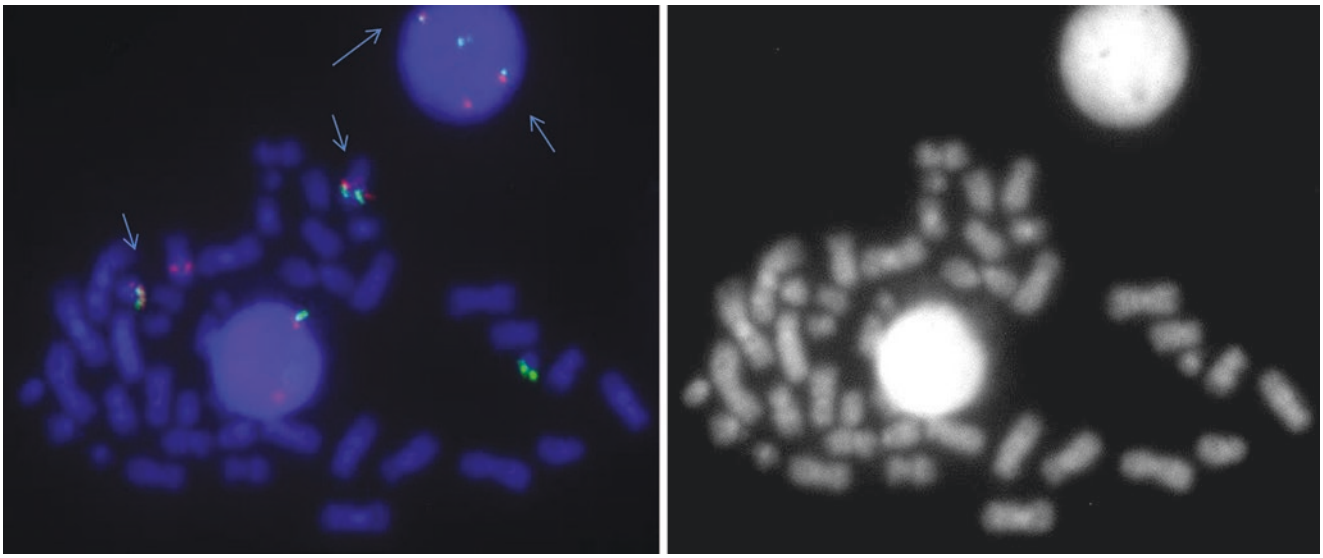


Fig. 23.1 FISH analysis confirms t(8;21) that results in the RUNX1/RUNX1T1 fusion in a patient with AML and t(8;21). FISH analysis employs dual color dual fusion probes with RUNX1 (21q22, labeled in green) and RUNX1T1 (ETO, 8q22, labeled in orange) and shows two fusions (arrow) on the derivative chromosomes 8 and 22, respectively. Normal single orange (RUNX1T1) and green (RUNX1) signals reside on normal chromosomes 8 and 21, respectively. The dual fusion signals are observed in interphase cells, too (arrows). The DAPI counterstaining (right) displays individual chromosomes concisely

APL with t(15;17)

t(15;17), the hallmark of APL, results in a fusion gene of the alpha retinoic acid receptor gene (*RARA, 17q21*) and the *PML* gene (15q24). The PML/RARA fusion has low sensitivity to retinoic acid in dissociation of a ubiquitous nuclear protein, i.e., N-CoR, which mediates transcriptional repression and thus leads to persistent transcriptional repression and prevents promyelocyte differentiation. This transcriptional repression can be efficiently overcome by treatment with ATRA, which leads to terminal differentiation of promyelocytes. With early treatment of ATRA, patients with APL have a very favorable prognosis.

There are several variant chromosome translocations of *RARA* in APL, such as the t(5;17) and t(11;17). APL with t(11;17) *PLZF/RARA* fusion does not respond to ATRA. Because APL is life-threatening due to high risk of hemorrhage, an early confirmation of APL is critical by rush FISH analysis within 2–4 h.

t(9;11) and Other KMT2A(MLL)11q23 Translocations

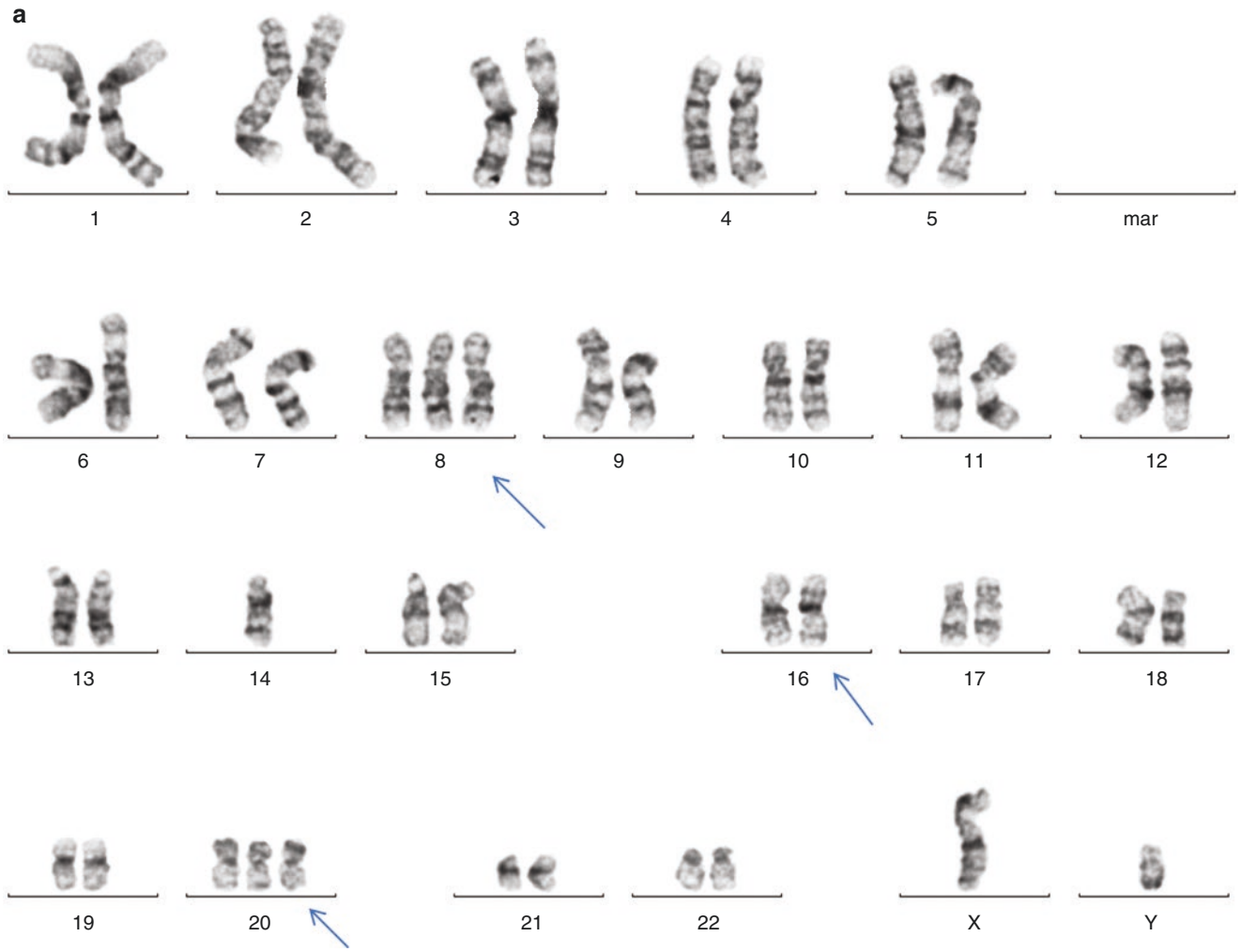
11q23/*KMT2A (MLL)* translocations are among most common chromosome translocations in both AML and ALL. So far, about 200 *KMT2A/MLL* fusion partner genes have been identified. In AML, t(9;11) and other MLL translocations are often associated with monocytic or monoblastic morphology (M5).

These translocations consistently disrupt the *KMT2A* gene (lysine [K]-specific methyltransferase 2A), which contains multiple functional domains, such as AT-hook DNA-binding motif, repression domain, and transcriptional activation domain. The protein has homology to the Drosophila trithorax gene, which regulates embryonic development and tissue differentiation. KMT2A methylates histone H3 lysine 4 (H3K4) and positively regulates expression of various *HOX* genes that are important in hematopoietic and lymphoid cell development. In *MLL* fusion gene, the activation domain at the 3′ region was replaced by the partner gene, thus, leading to loss of the H3K4 methyltransferase activity [12].

t(9;11) is associated with an intermediate prognosis in AML. All other 11q23/*KMT2A* translocations are associated with a poor prognosis in AML, such as t(6;11), t(10;11), and t(11;19). Notably, partial tandem duplication (PTD) of the *KMT2A* gene occurs in up to 90% of AML with +11 and is associated with poor prognosis in AML.

inv(3)/t(3;3)/MECOM/EVI1 Fusion

inv(3) or its variant t(3;3) are relatively rare in both AML and MDS, including therapy-related myeloid neoplasia, and are often associated with thrombocytosis and micromegakaryocytes [3, 13]. It results in the relocation of a distal *GATA2* enhancer closer to *MECOM/EVI1*, which activates *MECOM/EVI1* expression and simultaneously confers *GATA2* functional haploinsufficiency [14, 15]. The *MECOM* gene is a



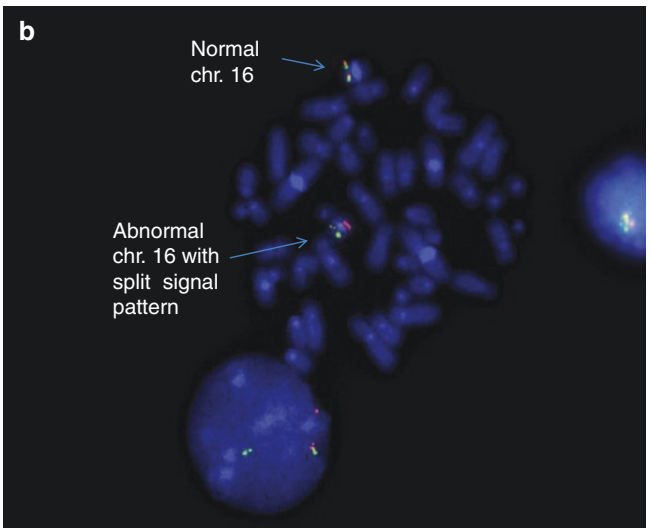


Fig. 23.2 inv(16)/CBFB/MYH11 fusion in a male patient with newly diagnosed AML detected by conventional chromosome analysis, FISH, and NGS fusion tests. (**a**) Karyogram shows a leukemia cell with inv(16) and gain of chromosomes 8 and 20 (arrows). The karyotype of all 20 metaphase cells is written as 48,XY,+8,inv(16)(p13q22),+20[20]. (**b**) FISH analysis using CBFB break-apart probes reveals an abnormal signal pattern with one intact fusion on the normal chromosome 16 and a split signal pattern on the derivative chromosome 16 (arrows). The probe franking the 5′ CBFB is labeled with orange, and the probe covering the 3′ CBFB is labeled with green. A split signal pattern was observed in an interphase cell, too. (**c**) Next-generation sequencing (NGS) of leukemia fusion studies confirms the presence of the CBFB/MYH11 fusion

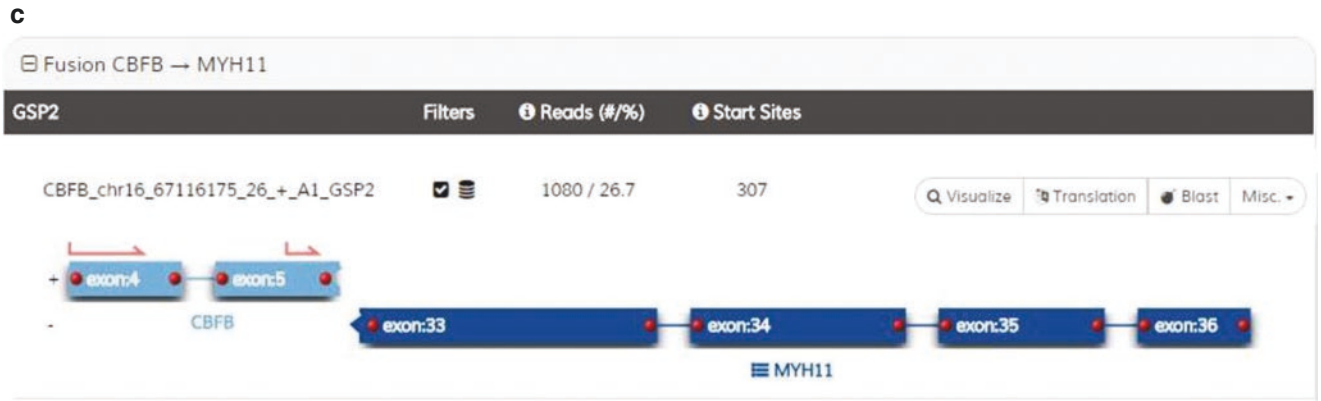


Fig. 23.2 (continued)

zinc-finger transcription factor gene that interacts with many important transcriptional and epigenetic regulators, such as CREBBP, GATA1, GATA2, DNMT3A, and DNMT3B, and thus mediates chromatin modifications and DNA hypermethylation. The activation of MECOM/EVI1 by inv(3)/t(3;3) promotes the proliferation of hematopoietic stem cells. In addition, the *MECOM/EVI1* gene is fused with *RUNX1* in t(3;21), often observed in t-MN and CML in blast crisis, which results in loss of *RUNX1* function and leads to overexpression of *MECOM/EVI1*. Inv(3)/t(3;3) is associated with a poor prognosis in AML and MDS. Loss of chromosome 7 is the most common secondary aberration.

t(6;9)/DEK/NUP214 Fusion

t(6;9) is a rare recurring chromosome translocation in AML and MDS and is often associated with basophilia and dysplastic morphology [16]. The translocation leads to a fusion between the *DEK* gene at 6p23 and *NUP214* at 9q34, which interferes normal nuclear transport. As in APL with t(15;17), and AML with a normal karyotype, FLT3 mutations, particularly FLT3 internal tandem duplications (ITD), are frequent in more than two thirds of patients with t(6;9) and likely contributes to an extremely poor prognosis in AML with t(6;9).

t(1;22)/RBM15/MKL1 Fusion

t(1;22) is a rare and unique chromosome translocation for acute megakaryoblastic leukemia (M7), most often in infants, not with Down syndrome. The translocation results in the *RBM15-MKL1*fusion [17]. The RBM15 and MKL1 are RNA- and DNA-binding motif proteins, respectively, which may modulate in chromatin organization, HOX-induced differentiation, or extracellular signaling pathways. There is no prognostic indication of this translocation.

Features of Massive Chromosomal Imbalances, Complex Karyotype, and Clonal Heterogeneity in AML

Besides the unique balanced chromosome translocation or inversion described above, many AML and high-risk MDS patients show a complex karyotype with multiple clonal evolutions, particularly in old adult patients, AML with myelodysplastic features, AML evolving from MDS or MPN, and therapy-related AML/MDS. These often include chromosome gain or loss and numerous structural abnormalities, such as del(5q)/−5, del(7q)/−7, del(17p)/−17, and +8 (Fig. 23.3). In these patients, mutations are common in genes involving in genetic and epigenetic pathophysiological pathways, such as myeloid transcription factors (RUNX1, CEBPA), activating mutations in signaling proteins (RAS, KIT, CBL, and FLT3), DNA methylation proteins (DNMT3A, TET2, IDH1/2, and SETBP1), chromatin modifiers (ASXL1, EZH2, KDM6A, KMT2A/MLL), and tumor suppressor proteins (TP53, WT1, and PHF6). In particular, TP53 deletion and mutation are among the most significant genetic abnormalities [18]. In these complex clones, chromothripsis seems the mechanisms that mess up complex chromosome aberrations, particularly for marker and ring chromosome and many derivative chromosomes [19]. At chromosome level, *chromothripsis* is featured by multiple chromosomal fragmentations due to a single event chromosome shattering over one or more chromosome regions. These complex genomic imbalances and heterogeneity are associated with very poor prognostic significance and poor response to treatment [22, 23].

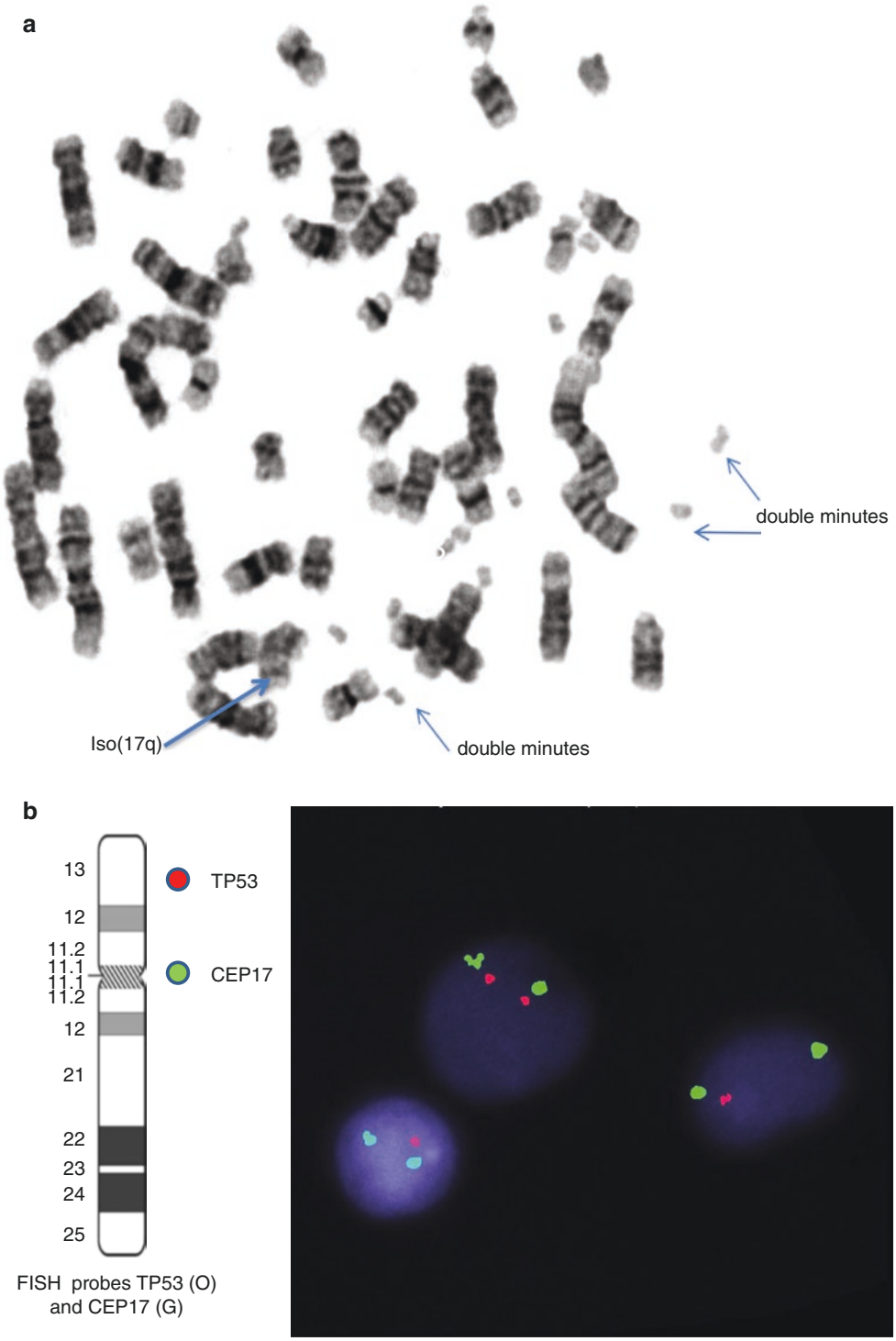


Fig. 23.3 Complex karyotype with loss of the short arm of chromosome 17, double minutes, and MYC amplification in a patient with AML. (**a**) A metaphase spread cell shows complex chromosome abnormalities, including gain of chromosome 4, abnormalities of chromosome 8 and 9, an isochromosome 17q (arrow), and numerous double minutes (arrows). (**b**) FISH analysis using double color probes for TP53 (17p13.1, labeled in orange) and the centromere probe for chromosome 17 (CEP17, labeled with green) (left, schema) showed two green signals and one orange signals in two interphase cells, i.e., loss of one copy of TP53, due to the isochromosome 17. A normal cell shows two orange and two green signals. (**c**) Genomic microarray tests revealed multiple complex chromosomal gains and losses on chromosomes 4, 8, 9, and 17. Amplification at 8q24 was observed, with copy numbers up to 36, including the MYC gene (arrow). (**d**) FISH analysis using MYC break-apart probes shows skyline colorful multiple signals all over the interphase nuclei, confirming the double minutes are MYC amplification in chromosome format, which is associated with poor prognosis in AML

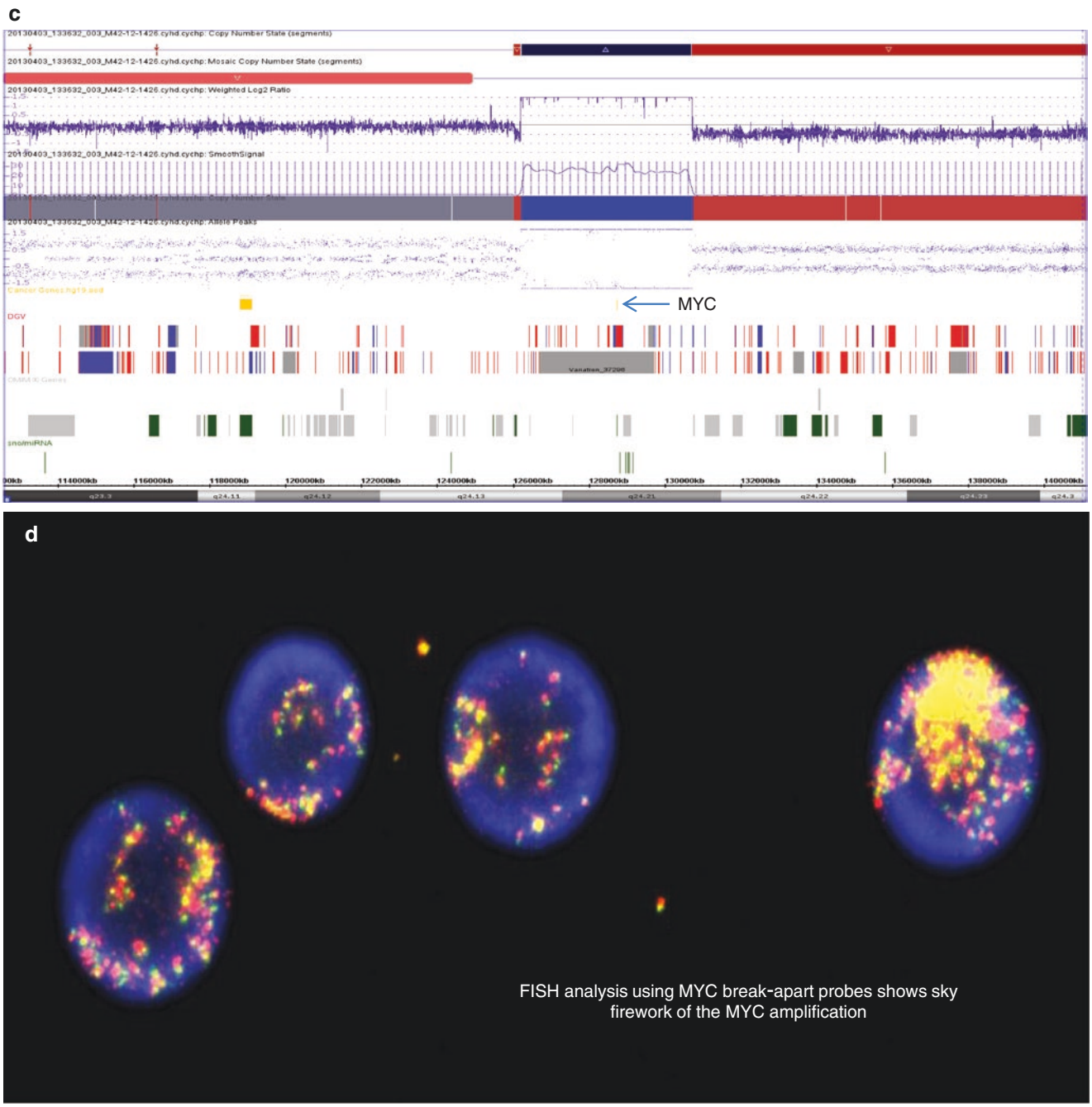


Fig. 23.3 (continued)

Myelodysplastic Syndrome (MDS)

In contrast to AML with many unique balanced chromosome translocations and inversion, myelodysplastic syndromes (MDS) are featured by various chromosomal loss, deletion, or gain, such as del(5q)/−5, del(7q)/−7, del(17p)/−17, +8, del(20q), etc., particularly in therapy-related MDS/AML. Only a few balanced chromosome translocations or inversions, such as inv(3)/t(3;3), t(3;21), and t(11;16), may be observed in MDS (Table 23.6) [3]. Chromosome abnormalities become more complex when MDS progress. Although these chromosomal deletion, loss, or gains are not specific for any subtypes of MDS, they carry significant prognostic impact in terms of patient survival and disease progression to AML and death and are critical in risk stratification and treatment selection (Table 23.7) [25]. Several genes in these

Table 23.6 Recurring chromosome abnormalities in MDS

Numerical or unbalanced structural abnormalities	Rare balanced chromosome translocations/inversion
+8	t(1;3)(p36.3;q21.2)
−7/del(7q)	t(2;11)(p21;q23)
del(5q)/t(5q)	inv(3)/t(3;3)(q21;q26.2)
del(20q)	t(3;21)(q26.2;q22)
−Y	t(6;9)(p23;q34)
i(17q)/t(17p)	t(11;16)(q23;p13.3)
−13/del(13q)	
del(11q)	
del(9q)	
idic(Xq)	
del(12p)/t(12p)	

Table 23.7 Prognostic significance of recurring chromosome abnormalities in MDS according to the Revised International Prognostic Scoring System (IPSS-R)

Cytogenetic abnormalities	Prognostic subgroups	Percentage
−Y, del(11q)	Very good	4%
Normal, del(5q), del(12p),del(20q), double with del(5q)	Good	72%
Del(7q),+8,+19,i(17q), any other single or double independent clones two or more unrelated noncomplex clones	Intermediate	13%
−7, inv(3)t(3;3)/del(3q), double with −7/del(7q), complex with 3 abnormalities	Poor	4%
Complex with 4 or more abnormalities	Very poor	7%

genomic lesions in MDS are involved in pathophysiology of various signaling pathway in normal and abnormal stem cell differentiation in leukemogenesis [28, 29].

Del(5q) and 5q- Syndrome

Del(5q) is among the most common chromosome abnormalities in de novo and therapy-related MDS. The deletion size is variable, most covering a large distal region of the long arm, i.e., 5q31 and 5q33. Deletions involving 5q31 are often observed in patients with high-risk and aggressive MDS, or with therapy-related MDS, and in many cases are part of a complex karyotype with *TP53* deletion and/or mutation, whereas a deletion of 5q33 is the hallmarker for the 5q- syndrome in the WHO classification, defined a cytogenetically isolated del(5q) or del(5q) with one other abnormality (excluding −7/del(7q)). 5q- syndrome primarily occurs in older women, age ranging from 65 to 70, and is associated with a relatively good prognosis, low risk of progression to AML and high treatment response to lenalidomide.

Several genes in these 5q deletion regions are implicated in leukemogenesis of MDS and AML, including *RPS14*, *EGR1*, *NPM1*, *APC* and *CTNNA1*, and microRNA *miR-145/146a*. *RPS14* at 5q32 participates in the maturation processing of 18S pre-rRNA, and del(5q) leads to its reduced expression and blockage of differentiation [26]. *APC*, *EGR1*, *NPM1*, and *CTNNA1* often involve in high-risk MDS with complex karyotype and *TP53* deletion or mutations. Various mouse models with deletion or loss of these genes display typical MDS phenotypes, indicative of a cooperative consequence of multiple genes in 5q deletion regions in pathogenesis of MDS.

5q- syndrome is associated with a good prognosis, whereas those with del(5q) as part of a complex clone with other chromosomal changes is associated with advanced MDS and a poor outcome.

−7/del(7q)

Loss of chromosome 7 or, less frequently, del(7q) is recurring abnormality in MDS, particularly in therapy-related MDS. Deletions often involve the middle region (7q22) or the terminal band (7q32–33) of the long arm. The *CUX1* gene at 7q22 likely is a conserved, haploinsufficient tumor suppressor by regulating target genes that promote hematopoiesis through distal enhancers by looping to target promoters [27]. Homozygous mutations of the *EZH2* gene at 7q32 are also frequent in MDS patients and result in loss of its histone methyltransferase activity. −7 is associated with unfavorable prognosis in both AML and MDS, whereas del(7q) as sole abnormality predicts an intermediate prognostic risk in MDS.

Gene Mutations in AML and MDS, Particularly with Normal Karyotypes

In addition to the recurring chromosome translocations, inversion, and chromosomal loss or deletion, numerous high-frequent gene mutations have been identified in AML and MDS, such as *FLT3*, *NPM1*, *CEBPA*, *BAALC*, *WT1*, *IDH1/2*, *KIT*, *and TP53*. Mutations of these genes provide critical prognostic significance and therapeutic selection in AML and MDS, particularly those with normal karyotype. *FLT3* mutations and *WT1* mutations are associated with poor prognosis, whereas *NPM* mutations, without coexistence of *FLT3* mutation, and biallelic mutations of *CEPBA* are indicative of a favorable prognosis in AML [24]. In MDS, most frequently mutated genes are spliceosome genes *SF3B1* and *SRSF2* and *TET2*, *ASXL1*, *DNMT3A*, and *RUNX1* [30]. These mutations often affect epigenetic regulation, i.e., DNA methylation and histone modification, RNA-splicing

machinery, transcription factors, and cytokine signaling pathways, and also carry important independent prognostic significance and associated with progression to AML. TP53 mutations occur in up to 15% of MDS and are more frequent in patients with therapy-related AML/MDS, particularly those with complex karyotype and/or monosomal karyotypes in AML and MDS, and are a strong predictor of poor prognosis [31]. Some of these mutations have been under intensive clinical trials with a promising outcome.

Features of Chromosome Abnormalities in Therapy-Related Myeloid Neoplasia (t-AML/MDS)

Therapy-related myeloid neoplasia (t-MN) is not rare in patients with history of Hodgkin lymphoma, non-Hodgkin lymphoma (NHL), breast cancer and other cancer, rheumatoid arthritis, or organ transplantation who received chemotherapy or immunosuppression treatment for these primary tumors or diseases. Clonal chromosome abnormalities are detected in up to 92% of patients with t-MN.

In general, two different recurring chromosome abnormality patterns exist and are associated with previous chemotherapy (Table 23.8) [20]. One is featured by del(5q)/−5, del(7q)/−7, del(17p)/−17, and/or complex karyotype and TP53 deletion, and many have *NUP98* rearrangement. This group is strongly associated with previous treatment with alkylating agents and/or radiation therapy. The other group is characterized by recurring chromosome translocations involving *MLL* or *RUNX1/AML1* and often noted in patients with previous treatment with DNA topoisomerase II inhibitors, such as VP16. These two categories are also associated with different clinical features and pathology findings (Table 23.8) [21].

Table 23.8 Features of cytogenetic abnormalities in therapy-related myeloid neoplasm associated with previous chemotherapy and disease outcome

Features of different t-MN	Alkylating agents related	Topoisomerase II inhibitor related
Chromosome abnormalities	del(5q)/−5	Translocations of MLL (11q23) and RUNX1 (21q22)
	del(7q)/−7	
	del(17p)/−17	
	Complex	
Preleukemia phase	MDS	Often none
Latency	5–7 years	2–3 years
Morphology	M1, M2, M4	M4, M5 most
Response to chemotherapy	Poor	Good
Long term outcome	Poor	Fair

Clonal Hematopoiesis of Indeterminate Potential (CHIP)

Clonal hematopoiesis of indeterminate potential (CHIP) refers to clonal hematopoiesis with associated somatic mutations, and diagnostic criteria of MDS or other hematologic neoplasms are not met [32]. The incidence of CHIP increases with age, and CHIP is associated with an increased risk for development of hematological cancers. Notably, mutations of *DNMT3A*, *ASXL1*, *TET2*, *SF3B1 and TP53* are common in both CHIP and MDS/AML. Clonal selection of these preexisting mutant clones in hematopoietic stem cells can be driven by oligoclonality associated with normal aging, inflammation, immune destruction, viral infections, defects in the bone marrow niche, and/or dysfunctional hematopoiesis, followed by clonal expansion by various hematopoietic stressors, which eventually progress to MDS and AML [33, 34].

Acute Lymphoblastic Leukemia (ALL)

B-Cell ALL

Chromosome abnormalities, including translocations, inversions, and genomic deletion or gains, occur in more than 80% of B-ALL. These aberrations have a significantly different incidences in pediatric and adults patients with B-ALL, with t(12;21) and hyperdiploid karyotypes most common in pediatric B-ALL, t(4;11) in infants patients, and t(9;22) in adults B-ALL (Table 23.9). As in AML and MDS, chromosome abnormalities play an important role in predicting disease prognosis, responding to chemotherapy, and selecting targeted treatment in B-ALL [35]. In addition, deletions or mutations of genes that regulate lymphocyte development

Table 23.9 Recurring chromosome abnormalities defining unique B-cell ALL according to WHO 2016 classification and their prognostic significance

Chromosome abnormalities	Genes involved	Clinical prognosis
t(9;22)(q34;q11.2)	BCR/ABL1	Poor
t(4;11)(q21;q23)	KMT2A (MLL)/AF4(MLLT2)	Poor
t(12;21)(p13;q22.3)	ETV6/RUNX1	Favorable
t(1;19)(q23;p13.3)	TCF3/PBX1	Intermediate
t(5;14)(q31;q32.3)	IGH/IL3	Poor
iAMP21/add(21q)/der(21q)	RUNX1?	Poor
Hyperdiploid (>50 chromosomes)		Favorable
Hypodiploid (<45) or haploid (1n)		Poor
t(8;14)(q24;q32)/t(2;8)/t(8;22)	IGH/IGK/IGL and MYC	Burkitt leukemia

are common in B-ALL, such as *PAX5*, *IKZF1*, *RB1*, *TP15/TP16 (CDKN2A/B)*, and *TP53* [36].

t(12;21)/ETV6/RUNX1 Fusion

t(12;21) occur in about 25% of children B-ALL and in about 3–4% adult patients with B-ALL. In chromosome analysis, it is cryptic so FISH analysis or RT-PCR is needed to detect. The translocation leads to a novel fusion of the *ETV6/*(*TEL gene* at 12p13.2) with the *RUNX1/AML1* gene at 21q22. RUNX1 is one of the CBF complex (see CBF leukemia with t(8;21)), and the ETV6 protein is a transcriptional repressor. The ETV6-RUNX1 fusion has dominant impact on the normal RUNX1function. The normal *ETV6* allele on the other chromosome 12 is frequently deleted. t(12;21) is associated with a favorable prognosis in B-ALL, with overall long survival and a low relapse risk.

t(8;14)/MYC/IGH Fusion

t(8;14) or its variants t(2;8) and t(8;22) are the hallmark of Burkitt leukemia and lymphoma, characterized by mature B-cell phenotypes. The translocations result in overexpression of the MYC protein due to the conjugation of the enhancer of *IGH*, *IGK*, or *IGL* to the *MYC* locus and are associated with a poor prognosis in B-ALL.

t(4;11)/AFF1/KMT2A Fusion and Other KMT2A/MLL Translocations

t(4;11) is the most common 11q23/MLL translocations in B-ALL and occurs in up to 60% of infant patients under 1 year old and in about 10% of adult B-ALL; the leukemia cells are often negative for CD10 expression. The t(4;11) results in a novel fusion gene of the *KMT2A* gene to the *AFF1/*AF4 gene at 4q21 and is strongly associated with a poor prognosis in both adults and children patients. The t(4;11) clones occur in uterus in many infant patients. In addition, other recurring 11q23/*MLL* translocations in B-ALL include t(11;19) with the MLL/ELL fusions and t(9;11) with MLL/AF9 fusions.

t(9;22)/BCR/ABL1 Fusion

t(9;22) occurs in about 30% of adult B-ALL patients, increasing to 50% of patients older than 50 and rare in children patients (up to 5%). As in CML, the t(9;22) results in the BCR/ABL1 fusion, with two different fusion transcripts due to different genomic breakpoints in BCR. One rare fusion protein is p210, which is identical in CML, with a break in major bcr region of *BCR*, and the other is p190 with a break in minor-bcr region of *BCR*. Both type fusion proteins lead to constitutive signaling via the RAS pathway of signal transduction and promote leukemogenesis.

t(9;22) is associated with an extremely poor prognosis in B-ALL, with poor responses to chemotherapy and short survival. Combined chemotherapy and tyrosine kinase (TK) inhibitors, such as imatinib, lead to promising improvement of outcome.

t(1;19)/E2A/TCF3 Fusion

t(1;19) is a common chromosome translocation in about 30% of B-ALL in children and rare in adult patients. Chromosome analysis revealed a balanced t(1;19) or, more frequent, unbalanced der(19)t(1;19). The translocation results in the *E2A*(19p13.3) and *TCF3* (1q21) fusion, which is associated with a favorable prognosis.

iAMP21

Intrachromosomal amplification of chromosome 21 (iAMP21) is detected by FISH analysis using probes for *RUNX1* in about 3–5% of B-ALL. Chromosome analysis often shows a complex structural abnormal chromosome 21. Microarray studies identify multiple gains and amplification along most of the long arm, with deletion of the subtelomeric region, likely due to a mechanism of breakage-fusion-bridge cycles, followed by chromothripsis. iAMP21 is associated with a dismal outcome, which may be overcome by intensive treatment of high-risk regimens.

Hyperdiploidy

Hyperdiploid clone with 55–56 chromosomes is frequently detected in about 30% of pediatric B-ALL and in 4–5% of adult B-ALL. A typical pattern of gain of chromosomes is those even numbered chromosomes, particularly chromosomes 4, 10, and 21, etc. Hyperdiploidy clone with more than 50 chromosomes, particularly with +4, +10, and +17, is associated with a good prognosis in B-ALL [37].

Hypodiploidy

Three groups of hypodiploid clones in B-ALL include (1) near-haploidy with 23–29 chromosomes, (2) low hypodiploidy with 33–39 chromosomes, and (3) high hypodiploidy with 42–45 chromosomes [37]. The near-haploid clone often have higher frequent alterations involving tyrosine kinase signaling and Ras signaling, whereas low hypodiploid clones are featured by high incidence of deletion and/or mutation of *TP53*, *IKZF2*, and *RB1*.

B-cell ALL patients with haploid or hypodiploid clones have a poor prognosis. A doubling of a near-haploid or low hypodiploid clone is common on B-ALL and masking as a hyperdiploid clone with a pattern of gains of two copies, rather than a single copy gain, of multiple chromosomes. It is critical to distinguish the true hyperdiploid clone that is associated with a favorable prognosis from masked doubling haploid or low hypodiploid clone. DNA index analysis and FISH tests are helpful, and genomic microarray with allele homo- vs heterozygosity may clarify these cases.

BCR-ABL1 (Ph)-Like B-Cell ALL

Ph-like B-cell ALL has no t(9;22)/BCR/ABL1 fusion but shares a similar gene expression profile to B-cell ALL with t(9;22). It occurs in about 13% of pediatric patients, 21% in adolescents, and up to 38% of adults with B-ALL and is resistant to standard chemotherapy and associated with a poor prognosis [35].

Various gene fusions and mutations occur in Ph-like B-ALL, commonly including *CRLF2* (cytokine receptor-like factor 2) translocations or overexpression, ABL1-class fusions, rearrangements of *EPOR* or *JAK2*. These genes usually involve in B-cell development, proliferation and differentiation, cell cycle regulation, and cell signaling [39]. Overexpression of *CRLF2*, due to a translocation with IGH [38], or fusion with *P2RY8*, resulting from an interstitial deletion in the pseudoautosomal region at the tip of the X and Y chromosomes, occurs in more than a half of Ph-like B-ALL. In the remaining non-CRLF2 Ph-like B-cell ALL cases, numerous translocations and mutation of several tyrosine kinase gene, such as *NUP214-ABL1*, *BCR-JAK2*, *STRB3-JAK2*, *IGH-EPOR*, and *EBF1-PDGFRB* fusion, and frequent mutations in *FLT3*, *CREBBP*, *IL7R*, or *SH2B3* (*LNK*), are common. Mutations of these genes result in constitutive kinase activation and signaling via activation of the ABL1 and JAK-STAT or RAS pathways, which are sensitive to tyrosine kinase inhibitors or JAK kinase inhibitors [40, 41].

T-ALL

Recurring chromosome abnormalities are detectable in about 50–70% of T-ALL, most commonly involving both T-cell receptor (*TCR*) and non-*TCR* gene loci (Table 23.10). The prognostic impact of most of these chromosome abnormalities in T-ALL is not defined yet [3, 42].

TCR Gene-Related Translocations and Rearrangements

Recurring chromosome translocations in T-ALL are those with *TRA/D* (14q11.2), *TRB* (7q34), and *TRG* (7p14). These translocations lead to deregulation, mostly overexpression of the partner genes that often are cell cycle inhibitor or a transcription factor. The most common transcription factor partner genes in these TCR receptor-related translocations are *HOX11* (*TLX1*, 10q24), *HOX11L2* (*TLX2*, 5q35), *MYC* (8q24), *TAL1*(1p32), *RBTN1*(*LMO1*, 11p15), *RBTN2* (*LMO2*, 11p13), *LYL1*(19p13), and *LCK* (1q34) [44].

Non-TCR loci-related chromosome translocations and deletion or gain are common, including deletion of the long arm of chromosome 6, the short arm of chromosome 9 (*CDKN2A/B*, *TP16*), MLL translocations, IGH translocations, t(10;11)/*PICALM-MLLT10* fusion, *NOTCH1and JAK1* mutations, and overexpression of *TLX1* (*HOX11*). Activating mutations of *NOTCH1* occur in more than a half of T-cell ALL, which may cooperate with other mutations in leukemogenesis of T-ALL [43, 45].

Table 23.10 Recurring chromosome abnormalities defining unique T-cell ALL according to WHO 2016 classification

Chromosome abnormalities	Genes involved
inv(7)(p15q34)/t(7;7)(p15;q34)	TRB/HOXA
t(1;14)(p32;q11.2)	TCRD/TAL1(SCL)
t(10;14)(q24;q11.2)	TCRD/TLX1(HOX11)
ins(10;11)(p13;q23q13)	KMT2A/MLLT10(AF10)
t(10;11)(p12;q14)	MLLT10(AF10)/ PICAL(CALM)
amp(NUP214/ABL1)	NUP214/ABL1
MYB amplification	
Hypodiploid (<45) or haploid (1n)	

Mature B- and T-Cell Non-Hodgkin Lymphoma (NHL) and Hodgkin Lymphoma

As in myeloid neoplasms, various recurring chromosome translocation, inversions, and deletion, loss or gain, and amplifications are detected in majority of B- or T-cell lymphoma, by using conventional chromosome analysis, FISH tests, microarray, and NGS studies. In contrast to those in myeloid neoplasms and many of B-cell ALL, these chromosome abnormalities often involve the immunoglobulin (IG) genes, most often the heavy chain gene *IGH* (14q32) or infrequently the light chain loci *IGK* (2p12) and *IGL* (22q11.2) in B-cell lymphomas, and the TCR loci, i.e., *TRA/D* (14q11.2), *TRB* (7q34), and *TRG* (7p14) in T-cell lymphoma. The most common chromosome translocations that are strongly associated with subtypes of B-cell lymphomas are t(14;18)/*IGH/BCL2* fusion in follicular lymphoma, t(8;14)/*IGH/MYC* fusions in Burkitt lymphoma, t(11;14)/*IGH/CCND1* fusions in mantle cell lymphoma (MCL), and t(11;18)/*API2/MATL1* fusion in mucosa-associated lymphoid tissue (MALT) lymphoma (Table 23.11) [46]. In contrast, the recurring chromosome translocations and inversions in T-cell lymphoma are relatively rare, and only a few subtypes of T-cell lymphoma show specific chromosome translocations or inversions, including t(2;5)/*ALK/NPM 1*fusion in ALK-positive anaplastic large T-cell lymphoma (ALTL), inv(14)/t(14;14) *TRA/D/TCL1* fusion in T-cell prolymphocytic leukemia (T-PLL), and isochromosome 7q, i.e., i(7q) in hepatosplenic T-cell lymphoma (Table 23.12).

Notably, none of these chromosome translocations are exclusively associated with any subtypes of lymphoma. t(8;14) or its variants t(2;8) and t(8;22) are the hallmark of Burkitt lymphoma and also frequently observed in diffuse large B-cell lymphoma (DLBCL), multiple myeloma, and CLL. Detection of t(11;14) is diagnostic in MCL and is also the most common chromosome translocation in myeloma. In addition, the emerging of recurring chromosome translocations is common in lymphoma progression or transformations, such as t(8;14) or *BCL6* translocations in DLBCL transforming from follicular lymphoma with t(14;18),

so-called double- or triple-hit high-grade B-cell lymphoma, which is strongly associated with a poor prognosis (Fig. 23.4) [47, 50]. *BCL6* at 3q26 is frequently observed in B-cell lymphoma, with various non-IG loci, such as *MYC*, *PAX5*, etc., which often lead to the replacement of the promoter of *BCL6* and overexpression of the *BCL6* gene [47].

In contrast to the recurring chromosome translocations in AML, which often results in a chimeric fusion gene that produces new fusion proteins with novel functions, most of the chromosome translocations in B- and T-cell lymphoma lead to the juxtaposition of the enhancer of the IG genes or TCR genes with the partner genes, which results in the overexpression of the partner genes, such as t(14;18) and the *IGH/BCL2* fusions [47].

In addition to these recurring chromosome translocations and inversions involving the immunoglobulin (IG) and T-cell receptor (TCR) loci, various recurring chromosomal deletion, loss and gain, or amplifications occur in most of B- and T-cell lymphoma. These include deletion of the long arm of chromosomes 6, 10, 11, and 13, deletion of the short arm of chromosome 17, and gain of the X chromosome and chromosome 3, 7, 12, etc. Amplification of the *MYC* gene (8q24) occurs in certain high-grade B-cell lymphomas. They are

Table 23.11 Recurring chromosome abnormalities in B-cell NHL

Chromosome abnormalities	Genes/chromosomes involved	Associated diseases
t(11;14)(q13;q32)/t(2;11)/t(11;22)	IGH/IGK/IGL and CCND1	MCL
t(14;18)(q32;q21)/t(2;18)/t(18;22)	IGH/IGK/IGL and BCL2	FL, DLBCL
t(3;14)(q27;q32)	IGH and BCL6	DLBCL
t(8;14)(q24;q32)/t(2;8)/t(8;22)	IGH/IGK/IGL and MYC	Burkitt, DLBCL
t(11;18)(q32;q21)	BRIC3/API2 and MALT1	MZBCL
t(1;14)(p22.3;q32)	IGH and BCL10	MZBCL
t(3;14)(p13;q32)	IGH and FOXP1	MZBCL
MYC and BCL2 and/or BCL6 translocations	MYC, BCL2, BCL6	Double- or triple-hit large B-cell lymphoma

MCL mantle cell lymphoma, *FL* follicular lymphoma, *DLBCL* Diffuse large B-cell lymphoma, *Burkitt* Burkitt lymphoma, *MZBCL* marginal zone B-cell lymphoma

Table 23.12 Recurring chromosome abnormalities in T-cell NHL

Chromosome abnormalities	Genes/chromosomes involved	Associated diseases
inv(14)(q11.2q32)/t(14;14)(q11.2;q32)	TRAD/TCL1A	T-PLL
t(2;5)(p23;q35) and variants	ALK/NPM1	ALK+ ALCL
i(7q)	Often with +8	Hepatosplenic T NHL
+3/+5/+X	Unknown	AITL

T-PLL T prolymphocytic leukemia, *ALCL* anaplastic large T-cell lymphoma, *AITL* angioimmunoblastic T-cell lymphoma

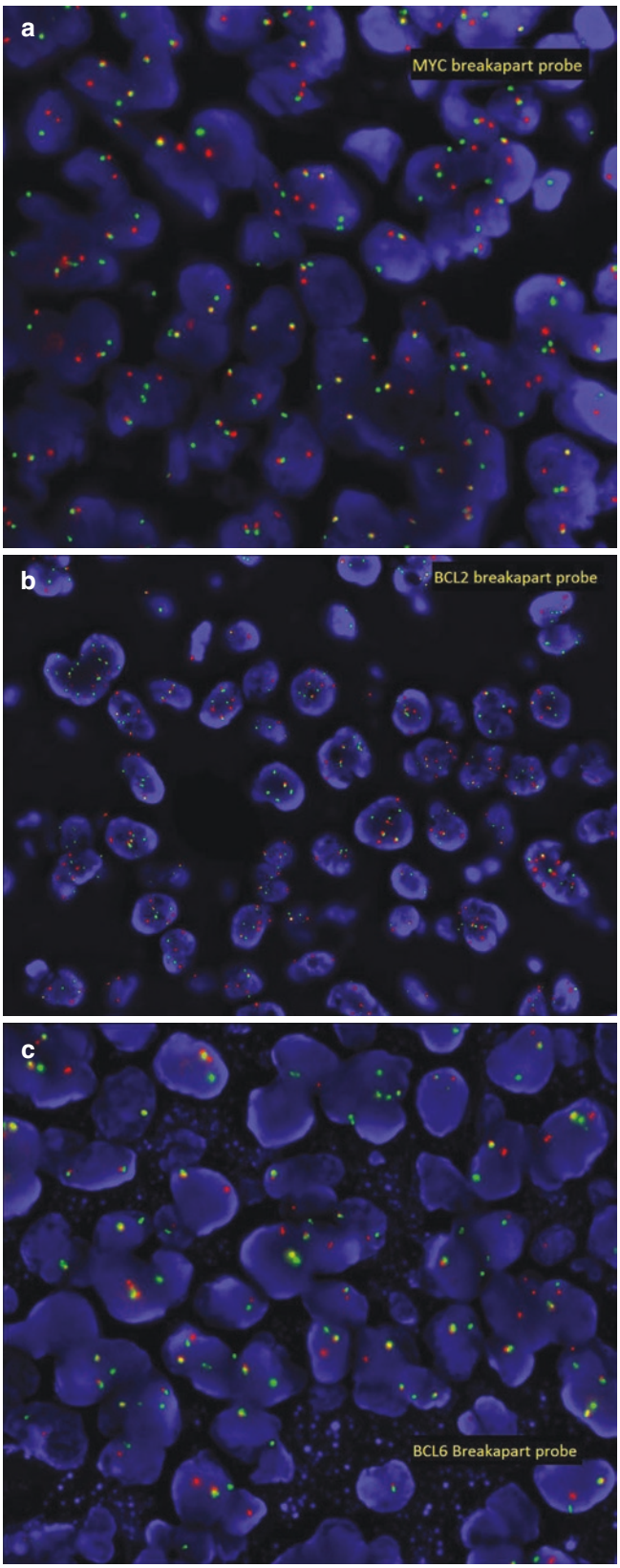


Fig. 23.4 Formalin-fixed paraffin-embedded (FFPE) FISH analysis reveals translocation patterns of MYC, BCL2, and BCL6 in a patient with diffuse large B-cell lymphoma. FISH analysis using break-apart probes for MYC (**a**), BCL2 (**b**), and BCL6 (**c**) reveals a split signal pattern for all three genes in most cells analyzed, indicative of a triple-hit high-grade B-cell lymphoma, which is associated with aggressive clinical course and poor prognosis

observed in various types of lymphomas and more often in advanced disease stages, along with complex and massive genomic abnormalities [49].

Non-Hodgkin Lymphoma

t(14;18)/IGH/BCL2 Fusion in Follicular Lymphoma and DLBCL

The t(14;18) is one of the most common chromosome translocations in B-cell lymphoma and is detected in up to 90% of follicular lymphoma (FL) and in about 30% of DLBCL. It results in a novel fusion of the *IGH* and *BCL2* genes, which leads to the overexpression of BCL2 due to the relocation of the enhancer of the *IGH* gene to the *BCL2* gene region. The *BCL2* protein is an inhibitor of apoptosis. Occasionally, t(2;18) or t(18;22), involving *IGK* or *IGL*, respectively, may occur [47].

In most patients, t(14;18) is accompanied by various additional chromosome abnormalities, particularly during the transformation of follicular lymphoma and in DLBCL. The most common secondary chromosome abnormalities include +X, +1q, +12p and duplication of the derivative chromosome der(18)t(14;18) and del(6q), del(10q). Many of these abnormalities may carry negative impact on the disease prognosis.

t(8;14)/IGH/MYC Fusion in Burkitt Lymphoma and DLBCL

The t(8;14) or its variants t(2;8) and t(8;22) are the hallmark of Burkitt lymphoma and also a common abnormality in DLBCL. In Burkitt lymphoma, t(8;14) is observed in about 85%, t(2;8) in 5% and t(8;22) in 10%. These translocations all result in the *IG/MYC* fusion that leads to the overexpression of MYC, which is a master in regulating cell cycle. In most cases with Burkitt lymphoma, t(8;14) often is sole or a part of a noncomplex clone, in comparison with those with t(8;14) in DLBCL. Additional chromosome abnormalities occur when the disease progresses, with most common +1q, +7, +12, and del(6q), del(17p), etc.

t(11;14)/IGH/CCND1 Fusion in Mantle Cell Lymphoma

t(11;14) is the hallmark of mantle cell lymphoma (MCL) and is critical in differentiating diagnosis with B-cell CLL, when the morphology and immunophenotypes of tumor cells are atypical. It leads to the conjunction of the enhancer of the *IGH* gene with the *CCND1* gene, which results in the expression of CCND1.

Additional chromosome abnormalities may occur in MCL with t(11;14), most common including deletion of 6q, 10q, 13q, and 17p and gain of chromosome 3, 12, etc. These secondary abnormalities are more frequent in disease progression, particularly in its blastoid variant, and have negative impact on prognosis.

t(11;18)/API1/MALT1 Fusion and Other MALT1 Fusion in Mucosa-Associated Lymphoid Tissue (MALT) Lymphoma

The t(11;18) is the primary chromosome translocations in MALT lymphoma and results in the *API2* (also called *BIRC3*)/MALT1 fusion. In addition, immunoglobulin gene-related chromosome translocations are detected in a small percentage of this entity, including t(14;18), which results in the *IGH/MALT1* fusions, and t(1;14) or its variant t(1;22), both involving the BCL10 gene (1p22) with either *IGH* or *IGK* gene. These translocations lead to the activation of the nuclear factor (NF)-kB activation pathway.

inv(14)/t(14;14)/TRAD/TCL1 Fusion in T-Cell PLL

The inv(14)(q11.2q32) or its rare variant t(14;14) is the hallmark of T-PLL, occurring in up to 90% of patients, and results in the *TRAD*(14q11.2)/*TCL1* (14q32) fusions. In rare cases, *TCL1* is fused with other TCR genes, i.e., t(7;14) with the *TRB/TCL1* fusion. In T-PLL without inv(14)/t(14;14), t(X;14) with *TRA/D/MTCP1* fusion is common.

i(7q) in Hepatosplenic T-Cell Lymphoma

An isochromosome of the long arm of chromosome 7, i.e., i(7)(q10), is the characteristic chromosome biomarker for hepatosplenic T-cell lymphoma (HSTL), which is a rare aggressive T-cell lymphoma predominantly in male young patients. Gain of chromosome 8 is the most common secondary chromosome abnormality in this entity.

t(2;5)/ALK/NPM1 Fusion in Anaplastic Large T-Cell Lymphoma (ALTCL)

T(2;5) is the hallmark of the majority (80%) of ALTCL, which is typically CD30 positive. The t(2;5) results in the *ALK*(2p23)/*NPM1*(5q35) fusion, which leads to constitutive tyrosine kinase activity of ALK activation [48]. There are at least seven other *ALK*-related translocations and inversion in ALCL that lead to ALK with other gene fusions, including common t(1;2)/*TPM3/ALK* fusion and rare t(X;2)/*MSN/ALK*

fusion, inv(2)/*ATIC/ALK* fusion, t(2;3)/*ALK/TFG* fusion, t(2;17)/*ALK/CLTC* fusion, t(2;17)/*ALK/ALO17* fusion, and t(2;22)/*ALK/MYH9* fusion. All ALK-positive ALCL have a favorable prognosis.

In the remaining ALCL patients with no *ALK* translocations, two new genetic markers have been identified recently, including the *DUSP22/IRF4* gene in t(6;7), which is associated with a comparable favorable prognosis as ALK-positive ALCL, and the TP63 gene rearrangements, which are associated with poor prognosis.

Hodgkin Lymphoma (HL)

Due to the rarity of the Hodgkin and Reed-Sternberg (H-RS) cells in lymph nodes, conventional chromosome analysis may reveal abnormal clones only in a small number of patients with HL, which often shows a complex karyotype of triploid or tetraploidy with multiple non-specific numerical and structural chromosome abnormalities. No specific chromosome abnormalities or genomic aberrations were identified in HL. However, applying FISH, microarray, and NGS studies, in combination with immunophenotyping of CD30, or microdissection of the H-RS cells has recently discovered several unique genetic aberrations in HL, including novel genomic amplifications, such as *PDL1/PDL2*; chromosome translocations, i.e., *CIITA*; [51] various gene mutations of the NF-kB; and JAK-STAT pathway, as well as epigenetic dysregulation. Some of these novel biomarkers are important therapeutic targets for genetic-based treatment, such as PD1 inhibitory antibody *nivolumab* in patients with refractory classical Hodgkin lymphoma (cHL) and PDL1/PDL2 amplification [52].

PDL1/PDL2 Amplification or Gain in cHL Integrative analysis with array and expression profiling reveals frequent immunoregulatory genes *PDL1/PDL2* amplification or gains as the target of 9p24.1 amplification (Fig. 23.5), including the JAK2 gene, which increased PD-1 ligand expression and further enhanced sensitivity to JAK2 inhibition in cHL and primary mediastinal large B-cell lymphoma (PMLBCL), which reveals the PD-1 pathway and JAK2 as complementary rational therapeutic targets. PDL1 over expression is associated with significantly poor PFS in patients with 9p amplifications [53, 54].

CIITA Abnormalities in HL The major histocompatibility complex (MHC) class II transactivator *CIITA* is frequently rearranged in about 15% of cHL and about 38% of PMLBCL. *CIITA* is often involved in various translocations that result in a novel fusion gene, which decreases MHC class II expression and increases PD-L1 and PD-L2 expression in these two entities (Fig. 23.6).

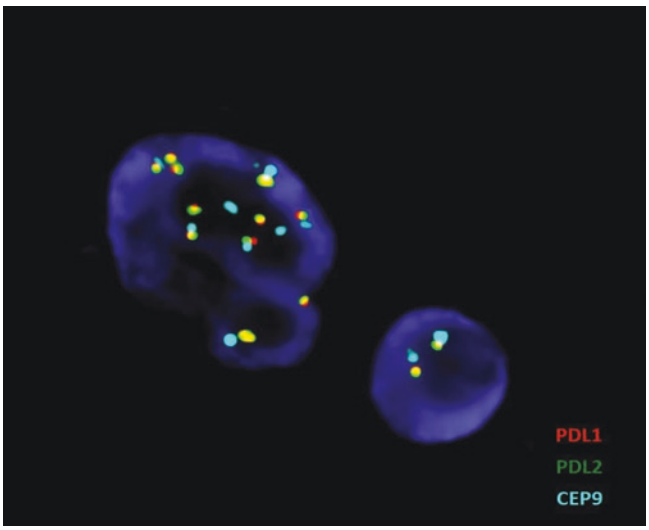


Fig. 23.5 FISH analysis of a patient with cHL showing amplification of PDL1 and PDL2 in a H-RS cell. PDL1 and PDL2 are labeled with orange and green color, respectively. The CEP9 centromere probe is labeled with aqua. The H-RS cell with bizarre nucleus shows multiple copies of PDL1 and PDL2 as well as CEP9

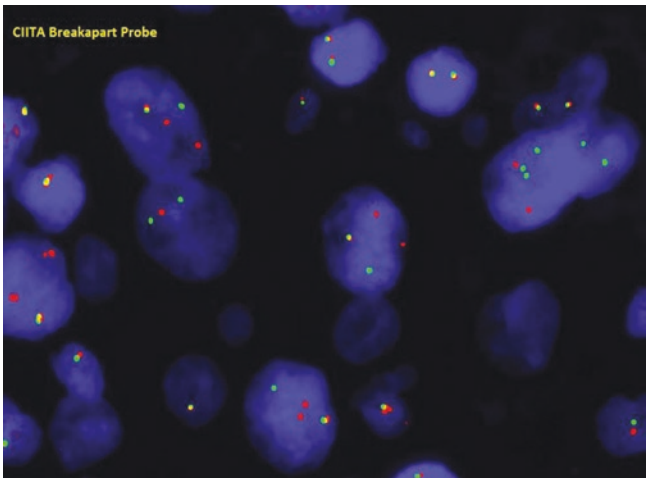


Fig. 23.6 FISH analysis of a patient with cHL showing CIITA rearrangement. The break-apart CIITA FISH probes are labeled in orange and green for the 5′ and 3′ regions, respectively. The abnormal cells show split signal patterns, indicative of a CIITA translocation

Chronic Lymphocytic Leukemia (CLL)

In about 80% of CLL patients, recurring chromosome abnormalities are detectable by conventional chromosome analysis, FISH, or microarray tests. The most common chromosome abnormalities in CLL are -13/del(13q), +12, del(6q)/MYB, del(11q)/*ATM*, and del(17p)/*TP53* (Table 23.13) [55].

Del (13q14) or, less frequent, −13 is the most common abnormality in CLL and can be detected in about a half of CLL cases. A heterozygous deletion is more common than a homozygous deletion. Two different deletions have been

Table 23.13 Recurring chromosome abnormalities in CLL and their prognostic significance

Chromosome abnormalities	Genes involved	Clinical prognosis
Del(13q)/−13	RB, miRNA-15/16	Favorable
+12	?	Intermediate
Del(6q)	MYB (6q23)	Intermediate
Del(11q)	ATM/BIRC3 (11q22.3)	Poor
Del(17p)	TP53(17p13.1)	Poor
Complex	?	Poor
T(14;19) (q32;q13)	IGH/BCL3	Poor

defined based on array studies; a large deletion involves the RB gene and the microRNA cluster of mir15, mir-16, and DLEU1/2 and is associated with an unfavorable prognosis, whereas the small deletion deletes the microRNAs only [57, 58]. The *RB* gene and mir15/16 and DLEU1/2 are negatively involved in the regulation of BCL2 mRNA expression, and thus, deletion of these microRNAs leads to overexpression of BCL2 in CLL. In 15% of CLL patients, +12 is detectable by chromosome and/or FISH tests. As sole abnormality, both del(13q) or −13 and +12 are associated with a relatively favorable prognosis in CLL.

Del(17p) is detected in about 10% of newly diagnosed CLL, and in about 50% of CLL with relapsed or refractory patients, and often results in loss of the TP53 locus. Del(11q), involving the ATM gene, present in about 20% of CLL patients. Both del(17p)/*TP53* and del(11q)/*ATM* are associated with poor prognosis in CLL with no respond to initial treatment well, an early relapse and short overall survival.

In addition to these recurring chromosomal imbalances above, several *IGH* translocations exist in CLL, including most commonly t(14;19), t(2;14), and t(14;18), which result in the fusion of IGH with *BCL3* (19q13), *BCL11A* (2p16), and *BCL2* (18q21) and deregulation of the relevant genes.

Moreover, high frequencies of several gene mutations have been identified in CLL, which involve in cellular signaling pathway, and are associated with poor prognosis. *NOTCH1* mutations occur frequently in CLL patients and are associated with unmutated IGVH, ZAP-70 expression, and a poor prognosis. The *SF3B1* gene is one of the spliceosome family genes that are responsible for splicing messenger RNA [56]. *SF3B1* mutations, particularly point mutations K700E are associated with a poor prognosis in CLL. Overexpression of miR-21 and miR-155 occur in a high frequency of CLL patients. MicroRNA-15a and miR-16-1 result in the upregulation of the BCL2 in CLL often without del(13q) [58].

Multiple Myeloma and Other Plasma Cell Neoplasms

Like other hematological neoplasia, multiple myeloma and other plasma cell neoplasms are featured by recurring chromosome abnormalities. In general, two relatively separated cytogenetic groups are identified; one carries various IGH translocations, and the other contains hyperdiploid clones, both often with recurring deletions of chromosomes 13 and 17 [59]. These chromosome abnormalities are associated with various prognostic significances and provide therapeutic guidelines (Table 23.14). In premalignant plasma cell proliferative disorder, i.e., monoclonal gammopathy of undetermined significance (MGUS), some of these recurring chromosome and genomic abnormalities also occur, indicative of their implication in the initiation of plasma cells neoplasms and in the progression to multiple myeloma [61].

Table 23.14 Recurring chromosome abnormalities in multiple myeloma and plasma cell neoplasia

Chromosome abnormalities	Genes/chromosomes involved	Clinical prognosis
Hyperdiploidy	+3, +5, +7, +9, +11, +15, +19, +21	Good
T(4;14)(p16;q32)	FGFR3/NSD2 and IGH	Poor
T(6;14)(p25;q32)	CCND3 and IGH	Good
T(11;14)(q13;q32)	CCND1 and IGH	Good
T(14;16)(q32;q23)	IGH and MAF	Poor
T(14;20)(q32;q12)	IGH and MAFB	Poor
T(8;14)(q24;q32)	MYC and IGH	Poor
Loss of 1p	TP15	Poor
Gain of 1q	CKS1B	Poor
Del(13q)/−13	RB	Poor
Del(17p)/−17	TP53	Poor

Similar to CLL, chromosome analysis in multiple myeloma is often limited due to low mitotic index of plasma cells in short-term culturing and only detects chromosome abnormalities in about 20% of myeloma patients. Genomic microarray tests on enrich plasma cell population are the choice of test for diagnostic myeloma specimen, along with FISH for IGH-related translocations. FISH tests are sensitive in monitoring specific abnormalities in follow-up samples and in detecting early progression such as del(17p) in small clones.

1. *IGH translocations*: There are five primary IGH translocations in myeloma, including t(4;14)/*FGFR3/IGH* fusion, t(6;14)/*CCND3/IGH* fusion, t(11;14)/*CCND1/IGH* fusion, t(14;16)/*MAF/IGH* fusion, and t(14;20)/*MAFB/IGH* fusion [59]. In addition, t(8;14)/MYC/IGH fusion often occur as secondary chromosome translocation in some myeloma patients. t(11;14) is the most common chromosome translocation in myeloma [60]. These IGH translocations result in the juxtaposition of the IGH active promoter region and overexpress to the partner genes, including transcription factors, growth factor receptors, and cell cycle mediators, and thus promote plasma cell growth and replication.
2. *Hyperdiploid clones*: In about a half of myeloma patients, a hyperdiploid clone is detected, typically with gain of the odd-numbered chromosomes, i.e., chromosomes 3, 5, 7,

9, 11, 15, 19, and 21. Hyperdiploid clones also result in overexpression of various genes on these chromosomes and promote cell proliferation.

3. *Del(17p/TP53) and other genomic gain or loss*: within these two major cytogenetic groups above, del(17p), including the *TP53* gene, and del(13q)/-13, as well as deletion and gain or amplification of the short arm and the long arm of chromosome 1, respectively, are recurring chromosome abnormalities in myeloma. In particular, del(17p/*TP53*) is more often detected in advanced disease stages and plasma cell leukemia and is strongly associated with poor treatment response and rapid disease progression [59].

Summary

Recurring chromosome translocations, inversions, deletion, loss or gains, and genomic imbalances are common in leukemia and lymphoma. Many of these genetic abnormalities are unique and significant for a precise diagnosis, risk stratification, and also importantly for predicting treatment outcome and prognosis. With novel molecular techniques, particularly next-generation sequencing, more genetic biomarkers are being discovered, many of which are genetic-based therapeutic targets that allow various clinical trials with promising novel treatment options, such as immune checkpoint inhibitors targeting PDL1/PDL2 in cHL. This progress benefiting from new genetic findings enables us to better understand the genetic mechanisms of relevant genes and novel fusions in various leukemo- and lymphogenesis and define new disease entities and allow us to study on treatment resistance in many patients even with novel genetic-based treatment.

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Understanding Molecular Testing in Patients Affected by Hematologic Disorders

24

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Starting Material for Molecular Assays

A variety of analyses are available for molecular testing as pathogenic alterations in the DNA of disease-causing genes often lead to detectable aberrations in RNA and protein expression and/or structure.

DNA is the most widely used molecular analyte. It is stable and can be easily extracted from a variety of specimen types, including fresh, frozen, and formalin-fixed paraffin-embedded (FFPE) tissues. Alterations within the human genome (complete set of DNA) are much better characterized than that of other molecular analytes (RNA and protein). As such, DNA is the preferred starting material for polymerase chain reaction (PCR), sequencing, and hybridization techniques. Although a more general view of the genome is obtained at a chromosomal level by conventional karyotyping, newer nucleotide-based technologies, including microarray and next-generation sequencing have enabled a more comprehensive view of the genome.

RNA is much more labile than DNA and can be quickly degraded in unprocessed or late to process hematologic specimens. Given its instability, for most molecular assays, reverse transcriptase is used to first convert RNA to complementary DNA (cDNA) to create a much more stable analyte (cDNA). RNA is the preferred analyte to detect fusion transcripts that occur in hematologic neoplasm. (The complexity of gene fusions makes their evaluation by DNA PCR-based techniques challenging.) RNA is also used to determine transcript expression, which cannot easily be garnered from DNA.

It is important to be aware of inhibitors of nucleotide-based testing that include acid decalcification and non-neutral buffered formalin fixatives that can severely damage nucleic acids. It should be noted that formalin-fixation and paraffin-embedding cause some damage to nucleic acids by formaldehyde-induced cross-linking, fragmentation, and deamination. Although these factors decrease the quantity and quality of DNA and RNA extracted, formalin-fixed paraffin-embedded (FFPE) tissues are in most cases adequate for nucleotide-based molecular testing.

Protein expression abnormalities are commonly assessed using immunohistochemistry of FFPE tissues, flow cytometry, or mass spectrometry (techniques not covered in this chapter).

Molecular Techniques

Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was developed in 1983 by Kary Mullis, who was awarded the Nobel Prize in Chemistry in 1993 for this work. PCR is the central technique used in molecular testing to amplify specific DNA sequences. These amplified DNA sequences can then be sequenced, sized, or labeled with probes to identify alterations in the DNA, including base pair mutations, insertions, or deletions. Many different PCR-based technologies are currently utilized in diagnostic testing as discussed in the next section.

PCR technique involves ~20 to 50 repeated thermal cycles of three steps: (1) denaturation, (2) annealing, and (3) polymerase extension that results in the exponential amplification of a targeted DNA sequence between two targeted DNA primer regions.

1. *Denaturation:* In the first step, target duplex DNA template is uncoupled into single strands by heat.

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2. *Annealing:* In the second step, two primers designed to bracket the targeted DNA sequence bind to the single-strand template DNA.
3. *Extension:* In the third step, DNA polymerases synthesize DNA along the template using combination of four dNTPs (Fig. 24.1).

The initial cycles of the PCRresult in exponential amplification of DNA product before gradual plateauing of the amplification rate. This plateauing is a result of the large amount of double-stranded template available in later cycles that favors re-annealing with itself at the expense of efficient primer binding and extension (Fig. 24.2).

PCR-Based Technologies

Reverse Transcription PCR (RT-PCR)

RT-PCR is a commonly used technique to examine target gene expression and detection of fusion transcripts.

RT-PCR technique is performed by initial production of complementary DNA (cDNA) from the target RNA template using reverse transcription. The cDNA synthesis commonly uses reverse transcription with non-specific priming that makes use of a mixture of random primers. This step is then followed by cDNA PCR amplification using targeted primers. RT-PCR can be combined with other techniques, such as quantitative PCR (qPCR), and this combined technique is referred to as real time or quantitative reverse transcriptase PCR (RT-qPCR).

Common Applications To detect gene fusions in neoplasms not easily detectable using DNA (i.e., *BCR-ABL1*, *PML-RAR*, *CBFB-MYH11*, *RUNX1-RUNX1T1*). Evaluation of fusions by routine PCR amplification of DNA can be very challenging as most gene fusions involve rearrangements of large and varied DNA sequences that commonly include large intronic (noncoding) DNA regions. The use of the RNA fusion transcript as a surrogate for the DNA rearrangement enables a more reliable PCR amplification procedure and provides direct assessment of amount of fusion transcript present. *BCR-ABL1* fusion gene is associated with formation of the Philadelphia translocation (Ph) and is one of the most common genetic abnormalities in chronic myeloid leukemia (CML) and adult acute lymphoblastic leukemia (ALL). Its presence is used to help diagnose and monitor response to treatment in CML and ALL [1]. Another example of a translocation detected by RT-PCR is the *ETV6-RUNX1* gene fusion frequently identified in childhood B-cell ALL patients that is considered a favorable cytogenetic subtype [2].

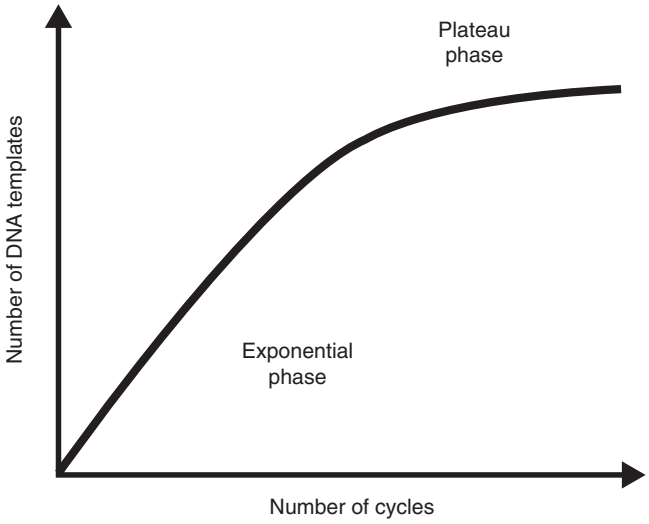

Fig. 24.2 Plateau effect in PCR

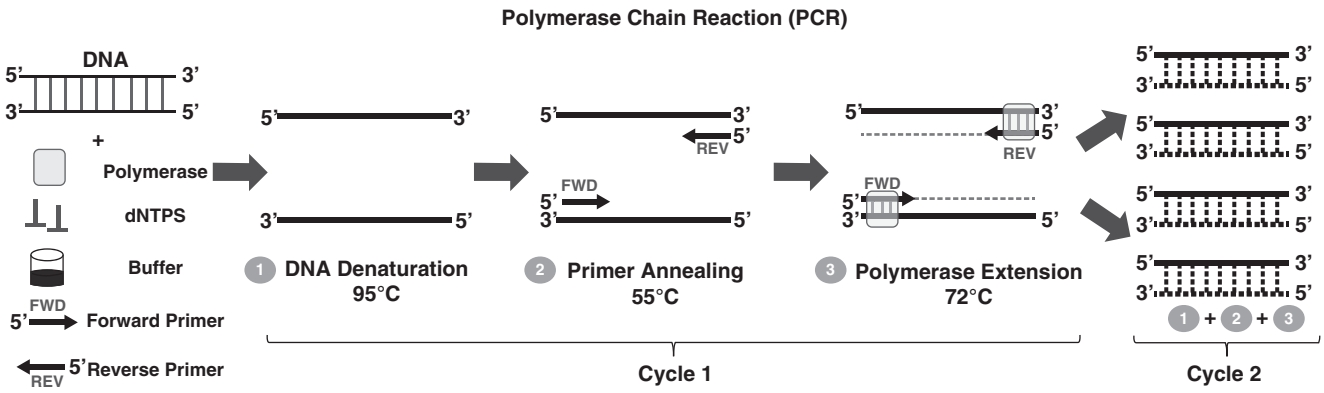

Fig. 24.1 Components of the conventional polymerase chain reaction (PCR) include a DNA template, deoxynucleotide triphosphates (dNTPs), DNA polymerase, and forward and reverse DNA primers. There are three stages in each cycle of a conventional PCR reaction: (1) DNA denaturation, (2) primer annealing, and (3) polymerase extension. In the exponential phase of PCR under ideal settings, each cycle results in doubling of the number of DNA templates. As shown, after two cycles there would be four copies of origin DNA template

Quantitative PCR (qPCR)

qPCR, also referred to as real-time PCR, makes use of fluorophore-labeled probes and initial exponential amplification rate of PCR to calculate the amount of DNA target present in the initial sample. qPCR is a widely used diagnostic technique because it is rapid and has a wide dynamic range of detection and a lower contamination risk (no post-PCR processing).

qPCR Technique A common qPCR design uses TaqMan sequence-specific fluorophore-labeled probes to measure the quantity of target PCR product in real time. During the annealing step of each PCR cycle, a specific TaqMan probe hybridizes to its target region. Next, during the extension step of each PCR cycle, the 5′ exonuclease activity of Taq polymerase cleaves the fluorophore from the probe resulting in fluorescence that is proportional to the degree of PCR product amplification. The amount of initial target is calculated by observing the PCR cycle in which fluorescence signal first becomes detectable (this threshold is called the Ct). For relative quantification, this observed Ct is expressed relative to co-amplified control DNA region (i.e., *ABL1* gene). This control amplified DNA (or amplicon) serves both as a reference standard for determining relative quantification and as an internal control. The quantity is represented as a relative ratio between target and control amplicons most commonly by the delta-Ct calculation. For absolute quantification, a stand curve determined from samples with known target copy number is used to convert the Ct to a copy number.

Common Applications Given its high sensitivity and quick testing time, RT-qPCR is used for detection of minimal residual disease, most commonly in quantitative monitoring of *BCR-ABL1* fusion gene in the setting of CML. In Ph+ patients, quantitative monitoring of residual disease is more valuable than qualitative detection to assist in clinical decision-making, especially when standardization in testing is implemented which ensures that results are comparable with previous tests. Other fusion transcripts monitored in leukemia by RT-qPCR include *PML-RARA*, *CBFB-MYH11*, and *RUNX1-RUNX1T1*.

RT-qPCR is also often used to assess relative RNA expression. For example, overexpression of *WT-1* mRNA has been recognized in a substantial number of acute myeloid leukemia (AML) patients. It has been shown to associate with prognosis and can be used as maker for minimal residual disease (MRD) [3].

Multiplex PCR

Multiplex PCR allows for more than one target sequence to be amplified concurrently in one PCR reaction mixture.

Multiplex PCR technique is performed by including multiple specific primer pairs in one reaction mixture. The design of specific primer sets is essential for a successful multiplex reaction. It is commonly combined with other PCR techniques such as RT-PCR and qPCR. In order to detect the specific signal associated with each amplified target, after PCR amplification, the DNA containing a mix of amplified sequences can be differentiated by (1) hybridization to fluorescent-labeled probes specific to different target sequences, (2) separated by size analysis (electrophoresis), or (3) sequenced by next-generation sequencing.

Common Applications Multiplex PCRs are utilized to detect multiple gene alterations at the same time. For example, different mutation variants of a gene that cause a neoplasm can be bundled in one multiplex PCR test. The reciprocal translocation of chromosome 9 and 22 has multiple potential combinations that generate the p190 (more common in ALL), p210 (more common in CML), and p230 isoforms. All three isoforms are detected using multiplex PCR: one PCR reaction with specific primers to each isoform.

Allele-Specific Oligonucleotide PCR or Amplification Refractory Mutation System (ARMS)

Allele-specific oligonucleotide PCR (ASO-PCR), also named as amplification refractory mutation system (ARMS), is a method for rapid detection of known single-based changes. This method differentiates DNA sequences with only one base pair difference. That base pair difference is referred to as a single-nucleotide variant (SNV). ASO-PCR is widely employed in clinical labs as it is simple, rapid, can be multiplexed, and is a very sensitive technique. The limitation with this technique is that prior knowledge of DNA sequence including differences between mutant alleles is required.

Allele-specific technique is performed by using PCR primers that are specific to either the mutant or wild-type sequence. The 3′end of one primer is designed to terminate at the polymorphic site, and extension/amplification will only occur if the allele-specific primer is bound to a completely complementary DNA template. This technique is combined with quantitative PCR (qPCR) to allow rapid comparison of amplification levels of either allele at a polymorphic site. In an alternate design, by adding outer primers, different alleles are distinguished by different PCR product sizes.

Common Applications Allele-specific PCR is used to detect single base pair mutations in neoplasms and hemoglobinopathies (i.e., β globin gene mutation in sickle cell anemia) and to determine haplotypes through identification of single-nucleotide polymorphisms (SNPs) [4]. Common targets in oncology include defining mutations such as (1) *JAK2* V617F (c.1849G>T) mutation which is a WHO major diagnosis criteria for myeloproliferative disorders and (2) *MYD88* L265P (c.794T.C) mutation which provides useful diagnostic value in the context of morphologic findings supporting lymphoplasmacytic lymphoma (LPL) [5].

Post-PCR Fragment Analysis or Sizing Assay (Capillary Electrophoresis)

Fragment analysis or sizing assay is a post-PCR technique that relies on differences in size of amplified DNA sequences.

Fragment analysis technique uses traditional gel electrophoresis or capillary electrophoresis (which has higher resolution) to determine the size of the PCR product (amplicon). Both techniques separate DNA molecules based on their size with the use of an applied voltage either through a porous gel or narrow-bore capillary.

Common Applications Genetic alterations that result in the change of target gene sequence length and are small enough to be reliably amendable to PCR (i.e., small insertions and deletions) can be detected by this method. Two of the most common applications include detection of *FLT3* internal tandem duplication (ITD) mutation and for determination of BCR/TCR clonality.

FLT3-ITD detection: FLT3-ITD mutation is seen in approximately one third of AML patients, and its presence is associated with a worse prognosis in cytogenetically normal AML [6]. As the name suggests, the *FLT3*-ITD mutation is a tandem (or repeat) duplication of DNA region which results in an insertion ranging from 3 to over 200 nucleotide base pairs in or near the juxtamembrane domain of the Flt3 receptor. Given the many potential mutations, *FLT3*-ITD mutations are commonly assayed using a sizing (fragment analysis) assay. PCR primers flanking the juxtamembrane coding sequence are used, and resulting amplified targets (amplicons) are separated using capillary gel electrophoresis. The PCR product of DNA template with a *FLT3*-ITD mutation would be larger (travel slower in capillary electrophoresis) due to insertion of the duplication sequence.

Clonality (BCR/TCR rearrangement) analysis: During B- and T-cell development, the germ line VDJ fragments of the immunoglobin and T-cell receptor (TCR) genes are rearranged by random deletion or insertion of nucleotides to generate unique sequences in each lymphocyte clone. Clonality is one of the considerations that may help distinguish malignancy from a benign lymphoid proliferation. PCR primers are designed to bracket the different VDJ rearrangement areas, and different size of PCR product indicates rearrangement. Multiplex PCR is used to cover all possible VDJ rearrangement sites. In a "normal" or polyclonal population, multiple bands are seen in a Gaussian distribution of sizes as would be expected by many random events. In cases where there is monoclonality present, normally one prominent peak or band is seen for a VDJ rearrangement site. This type of analysis is not quantitative and does not provide sequence information. As such, technical or biological pitfalls (i.e., selective amplification or pseudoclonality) can at times lead to false-positive results for clonality.

PCR-Restriction Fragment Length Polymorphism (RFLP)

A restriction enzyme (also known as an endonuclease) is an enzyme that cuts DNA at specific nucleotide sequences (restriction sites). Restriction enzymes are widely used as DNA editing tools in molecular cloning and gene modification. Restriction fragment length polymorphism (RFLP) analysis refers to the technique whereby DNA samples are digested with restriction enzymes and the resulting DNA fragments separated by electrophoresis based on length differences. If mutations are present at the restriction sites, a different DNA fragment profile will be seen.

Application of RFLP alone is limited by large sample amount and the complexity of probe labeling. More commonly, amplified target region by PCR is subject to RFLP analysis that enables identification of known mutations (sequences) that contain restricted sites. Amplified PCR products are first subjected to restriction enzyme cleavage and then analyzed for length by electrophoresis. DNA sequences matching predesigned restriction sites will result in cleavage and thus multiple or shorter fragments.

Common Applications Based on primer design and selection of restriction enzyme, RFLP analysis is used to assess a variety of DNA alterations. It also can be combined with other techniques such as short tandem repeat analysis to identify microsatellite variants. One example is the analysis for a single point mutation at nucleotide c.1691 (G>A) in exon 10 of Factor V gene that is a cause of Factor V Leiden.

High Resolution Melting Analysis

High resolution melting (HRM) analysis is a post-PCR analysis method to identify variations in nucleic acid sequences. After PCR, the amplicon cDNA is heated gradually and precisely from 50 to 95 °C. Once the melting temperature of the double-strand DNA is reached, the two DNA strands separate. By using a dye that only binds to double-stranded DNA, this process can be monitored and plotted (melting curve) in real time. The presence of a mutation or variant in the DNA alters the melting temperature shifting the melting curve.

This method is commonly used in SNP genotyping or point mutation detection.

Short Tandem Repeat (STR) Analysis

A short tandem repeat (STR) is a repeated DNA sequence motif consisting of two to seven base pairs. There are thousands of STRs throughout the genome. At a single locus, multiple alleles are possible depending on the number of repeats. Each individual is expected to have unique STR profile. Short tandem repeat is amplified by PCR because they are bracketed by unique sequences. Different alleles are identified based on the length of PCR product by capillary electrophoresis or gel electrophoresis. STR analysis is wildly used in forensic analysis and population genetics.

Common Applications STR analysis is most commonly used in chimerism (engraftment) analysis after bone marrow transplantation. It is one of the parameters used to help determine disease relapse or graft rejection. High STR variability in the population makes it ideal for unique identification of individuals (donor vs. recipient). Both pretransplant recipient and donor sample are required for comparison in this type of analysis.

Gap-PCR

Gap-PCR is used to help detect larger deletions (>2 kb) that due to their large size are technically difficult to amplify using conventional PCR. Multiplex gap-PCR can be used to detect multiple pathogenic deletions simultaneously.

Gap-PCR technique uses specific primer sequences designed to flank a known deletion. Since the primers are specific to flanking sequences that are brought close together only in the presence of the deletion, PCR amplification should only occur in the presence of the deletion. As a control for the wild-type sequence, a primer that anneals to the deleted sequenced can be used to generate an additional product with one of the flanking primers. This internal control serves to reduce the likelihood of a false negative in the wild-type and will indicate if an individual may be heterozygous for the deletion. The presence of the PCR product can be detected by electrophoresis or high resolution melting analysis.

Common Applications Gap-PCR is commonly used for simple and rapid detection of common deletions leading to reduced or impaired production of alpha globin gene resulting in α-thalassemia. The most common mutations in α-thalassemia are gene deletions that can be evaluated using multiplex approach with primers specific for multiple types of deletions [7]. This assay is limited to deletions with known breakpoints. Less common globin disorders caused by deletions in delta-beta globin gene (resulting in hereditary persistence of fetal hemoglobin and hemoglobin Lepore) and in beta-globin gene (resulting in Filipino- and 619 bp-deletion β^0-thalassemia) also are detected by gap-PCR.

Inverse Shifting-PCR (IS-PCR)

IS-PCR is a method that allows for rapid detection of known structural rearrangements, including large duplications, inversions, and deletions, using PCR to amplify DNA with only one known sequence.

Inverse shifting PCR technique involves three main steps: (1) restriction enzyme digestion, (2) self-ligation, and (3) PCR with post-analysis. It is initiated with a restrictive endonuclease digestion and self-ligation to form a circular DNA ligation product including the known insertion site. Ligated circular DNA corresponding to wild-type sequence and that with a known large inversion or duplication are differentiated by using PCR with primers complementary to known internal sequences that are specific to the wild-type or altered sequence. Most commonly, the resultant PCR products (of predicted varying sizes) are differentiated by gel or capillary electrophoresis.

Common Applications Hemophilia A is an X-linked inherited bleeding disorder caused by mutations in the gene encoding coagulation factor VIII. Approximately 50% of severe hemophilia A cases are attributable to gene inversions in Factor VIII introns 1 and 22. Inversion PCR method can be used for carrier testing and prenatal diagnosis for hemophilia A in the appropriate clinical context [8].

Multiplex Ligation-Dependent Probe Amplification (MLPA)

MLPA is a type of multiplex PCR technique able to detect difference in copy number or to distinguish known single-nucleotide variants (sequences differing in only one nucleotide). In comparison with multiplex PCR where the target sequences are amplified, in MLPA, it is the probes that hybridize to target sequences that are amplified. In this way, a single universal primer pair can be used with many different-sized probes to assay many different DNA sequences simultaneously. This multiplex technique is fast, accurate, and can detect small deletions missed by other techniques.

MLPA technique encompasses four main steps: (1) DNA denaturation and hybridization of MLPA probes, (2) ligation reaction of two adjacent probes, (3) PCR reaction, and (4) separation of amplicons.

1. *Denaturation and hybridization*: DNA is denatured and hybridized with MLPA probes. MLPA probes consist of a pair of probes designed to hybridize (bind) the targeted sequences immediately adjacent to each other. The MLPA probes pair can be (1) fluorescently labeled, (2) tagged by different sized "stuffer" sequences (to produce different

product sizes for each different probe pair), or (3) tagged by sequences that can be used to separate different probes on beads.
2. *Ligation reaction*: Only when the two probes bind (hybridize) to adjacent targets can a ligation reaction take place between probes.
3. *PCR* reaction using universal primers attached to each hybridized probe will amplify the product. Only ligated probes will be exponentially amplified in the PCR reaction.
4. *Separation of amplification products* is normally performed by capillary electrophoresis. However, other methods can be used depending on how the MLPA probes are tagged.

Using multiple pairs of target-specific probes that can all be amplified by the same primer multiplexes this assay. The sequences are simultaneously amplified with one primer pair and result in a mixture of PCR products, in which each PCR product of each probe pair has a unique characteristic (i.e., length, if different length probes or stuffer sequences are used). The resulting amplification products can be separated (i.e., for length by capillary electrophoresis) and compared to a pattern obtained from a reference sample. The amount of amplification of each product (i.e., peak height in capillary electrophoresis analysis) reflects the relative copy number of that target sequence.

Common Applications MLPA is commonly used for copy number detection, methylation quantification, or combined with reverse transcription to enable RNA profiling. It can also be used to detect known point mutations, as probes can be designed to detect the wild-type or mutated sequence. Many known pathogenic mutations lead to alpha-thalassemia, and MLPA testing is commonly used because it can detect several of the most common mutations in one reaction [9].

Digital PCR

Digital PCR was first described by Bert Vogelstein and Kenneth Kinzler [10]. This relatively new technique was developed to offer more sensitive mutation detection and precise quantification. Digital PCR works by partitioning a sample (template DNA) into many individual parallel PCR reactions. The results of those reactions are scored as positive or negative based on detection of fluorescent-labeled probes designed to hybridize (bind) to mutations of interest. These binary results are fitted to a Poisson distribution model to provide an absolute quantification that does not require calibration curves (as required in qPCR for absolute quantification). Another shortcoming of qPCR is that the sensitivity of detection is limited by background competition from the wild-type templates in the PCR reaction. In digital PCR technique, PCR reactions are partitioned into individual reactions with one or no template alleviating the background competition. Digital PCR is an extremely sensitive assay that can provide precise quantification without the need of a standard curve or certified reference material.

Digital PCR Technique works by distributing temple DNA into thousands to millions of nanoscale compartments to have either a single or no template molecule. Distribution of DNA template into nanoscale compartments is accomplished by emulsion (droplet digital PCR, i.e., Bio-Rad and RainDance PCR systems) or by carrying out PCR in nanoscale reaction chambers (i.e., QuantStudio3D by Life Technologies). PCR reaction is then performed independently in each compartment. After PCR amplification, each PCR reaction is scored as positive or negative (normally using sequence-specific fluorescent-labeled probes). This binary result gives the technique its "digital" name. The absolute quantity of specific target DNA molecules is determined by simple summation of all reactions and compared to amount of droplets with normal (wild-type) DNA.

Common Applications Digital PCR is utilized for greater sensitivity and precision in detecting minority targets such as in the setting of monitoring minimal residual disease (MRD). For example, for higher sensitivity and precision than in qPCR, *BCR-ABL1* quantification in CML MRD monitoring can be performed by digital PCR [11]. Currently, further studies are needed to determine how differences in the scale of detection between conventional quantitation and digital PCR could potentially impact the clinical management of patients in an MRD setting.

Chromosomal Analysis (Karyotype)

Chromosomes were first visualized in the late nineteenth century, and their analysis has been a part of clinical practice for over 50 years. In order to understand the methods used to detect disease-related molecular genetic changes, one must first understand the basic structure of chromosomes. The human chromosome complement includes 23 pairs of chromosomes, with 22 autosomes and one set of sex chromosomes. The autosomes are numbered essentially in order of size. Each chromosome contains a centromere, which breaks the chromosome into two arms. The shorter arm is denoted p and the longer arm q. The two arms may be nearly identical in size (metacentric), or the centromere may be skewed toward one end (submetacentric) or to the extreme end (acrocentric) with very little genetic on the p arm. The ends of the chromosomes are capped by telomeres, which contain repetitive DNA sequences thought to bring stability to the chromosome.

Karyogram is the ordered depiction of an individual's chromosomal composition. The chromosome complement itself is called the *karyotype* and is expressed as the number of chromosomes followed by the sex chromosome makeup.

Any structural abnormalities are listed thereafter. For example, a normal male karyotype is written 46, XY, whereas a male with an extra chromosome 6 is denoted 47, XY,+6. Important nomenclature for various structural abnormalities is listed in Table 24.1 (Fig. 24.3).

Cytogenetic Technique A well-prepared cytogenetic slide will have the chromosomes of a single cell in close proximity to one another and yet spread out enough to clearly visualize each chromosome and its banding pattern. Classical cytogenetics generally involves counting at least 15–20 sets of metaphase chromosomes. Under special circumstances, such as mosaicism or discrepant results, additional chromosomes will be counted. In metaphase, the genetic material is most condensed and is therefore most easily observed. In order to produce *metaphase chromosomes*, a patient sample is cultured to allow cell division. Bone marrow samples may undergo mitosis in culture media. However, other specimens such as lymphocytes require a substance that induces mitosis (a mitogen, phytohemagglutinin is commonly used) in order to divide. Some cancer cell types (i.e., myeloma cells) do not grow well in culture, making it difficult to obtain metaphase

Table 24.1 Types of and abbreviations for chromosome abnormalities

del	Deletion	Loss of a chromosomal region (partial monosomy); may be terminal or interstitial
dup	Duplication	Additional copy of a chromosomal region (partial trisomy); may be terminal or interstitial
ins	Insertion	Addition of chromosomal material
inv	Inversion	Reversal of normal gene arrangement of a chromosomal segment; pericentric- breakpoints on either side of centromere, paracentric- both breakpoints within same arm of chromosome
i	Isochromosome	Two copies of one chromosome arm without the other arm
mar	Marker	Distinct accessory chromosome fragment
r	Ring	Circularized chromosome with loss of telomeres
t	Translocation	Rearrangement between two different chromosomes produces two derivative (der) chromosomes that are hybrids of the two involved chromosomes

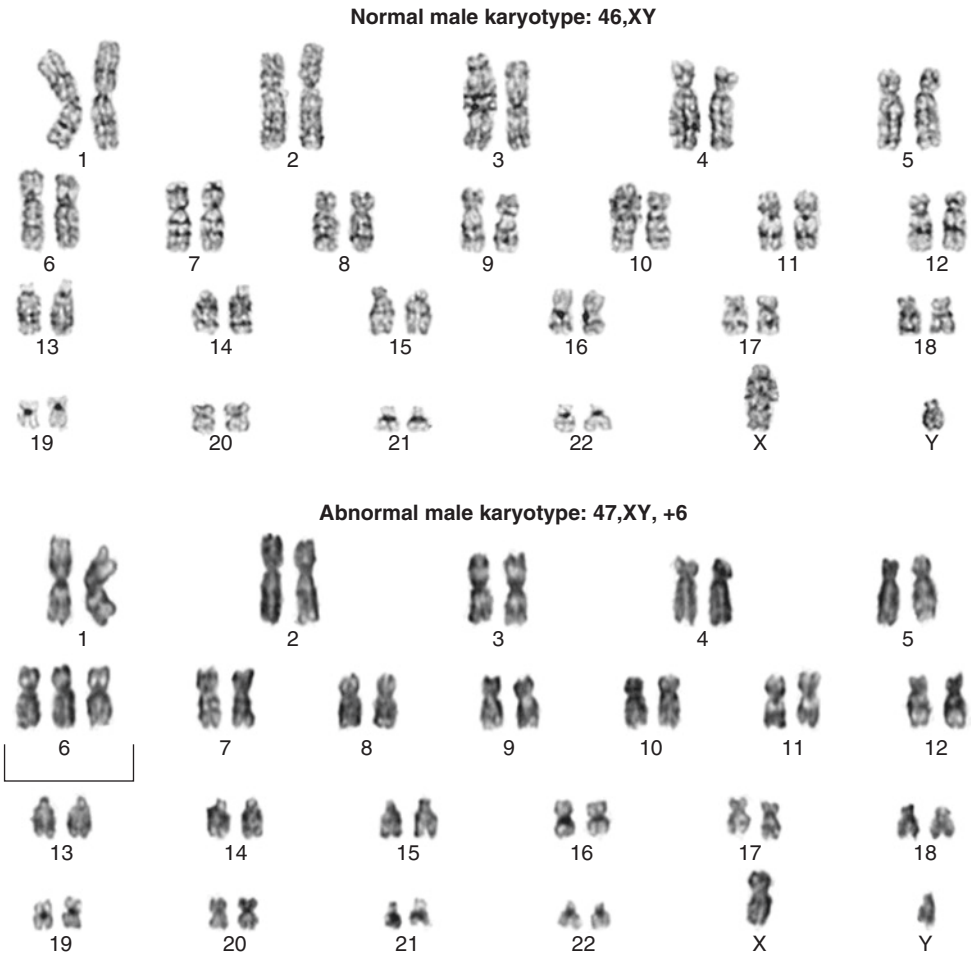

Fig. 24.3 G-banded karyotypes of a normal male karyotype and abnormal male karyotype with trisomy 6 (three copies of chromosome 6). (Images courtesy of Dr. Shashirekha Shetty)

chromosomes. Culture may take from 2 to 5 days, depending on cell type under analysis. Cells may then be treated with a drug to arrest the cells in metaphase. They are then "dropped" and affixed to a slide, stained to give the characteristic banding pattern, and viewed under the microscope to produce the final karyotype. Banding is achieved using DNA-binding dyes that give reproducible banding patterns based on the nucleotide composition of each chromosome region. Common staining protocols include G-banding (Giemsa stain), R-banding (gives reverse pattern of Giemsa), and C-banding (stains centromeres). Whereas in the past chromosomes were cut and pasted together from black and white photographs, computer software is now available to assist in the preliminary ordering of chromosomes. The software makes a preliminary karyogram, which is then confirmed or corrected by a cytogenetic technologist.

In hematology, the clinician is likely to encounter somatic changes that may occur in only a fraction of the cells counted in a karyotype. In this context, the different karyotypes found are reported in decreasing frequency. A man found to harbor the Philadelphia chromosome in 75% of his bone marrow cells would have the karyotype: 46,XY,t(9;22)(15)/46,XY(5) which denotes (15 abnormal cells with the translocation and 5 normal male cells).

Common Applications Chromosome analysis is important in diagnosis, risk stratification, disease monitoring, and therapy decisions in diseases including AML, CML, ALL, myelodysplastic syndromes (MDS), non-Hodgkin's lymphoma, plasma cell myeloma, and chromosome breakage syndromes. While newer technologies have become clinically relevant due to their increased analytical sensitivity or ability to detect smaller genetic abnormalities, classical cytogenetics remains the most commonly accepted clinical technique that provides structural information about the entire genome. More recent techniques including fluorescent in situ hybridization (FISH), DNA sequencing, and array technologies allow detection of alterations not detected by conventional cytogenetics, including cryptic rearrangements or non-chromosome scale changes (such as single base pair mutations, small deletions, or copy number changes across the genome). Although these new technologies currently serve as complementary approaches to cytogenetics in clinical medicine, there is an increasing promise that they may play a more primary role in the future.

Fluorescent In Situ Hybridization

Fluorescent in situ hybridization (FISH) is a technique used to visualize the presence, absence, amplification, splitting, and/or recombination of specific genetic sequences using unique DNA probes that are labeled with a fluorescent signal. While metaphase FISH is analogous to classical cytogenetics with the DNA probes replacing banding dyes, FISH can also be performed on cells regardless of cell cycle phase. So-called interphase FISH does not rely on cell division and therefore can be applied to any cellular sample, including difficult to culture cell types. Furthermore, because there is no culturing step, interphase FISH can be completed more quickly than classical cytogenetics. One example of the clinical utility of this faster turnaround time is in testing for the t(15;17) translocation to confirm the diagnosis of acute promyelocytic leukemia (Fig. 24.4).

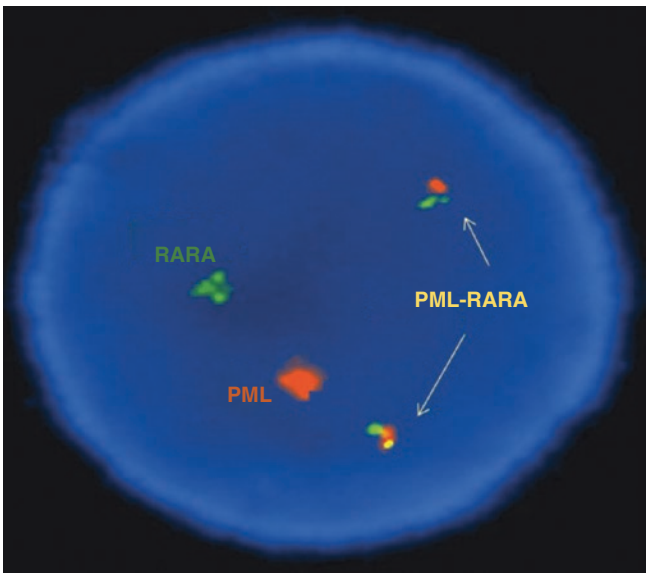

Fig. 24.4 Florescence in situ hybridization (FISH) for detection of PML/RARA translocation. Shown are results for interphase FISH using LSI PML/RARA dual color dual-fusion translocation probes. This test employs two large probes spanning the breakpoints for the two involved genes. In this positive cell, one signal each for the wild-type alleles (PML and RARA), and two fused signals (PML-RARA) are generated

FISH Technique Cells are placed on a slide and fixed in formaldehyde. The chromosomal DNA is then denatured by heat. Specific probes with attached fluorescent labels are then hybridized to their complementary sequence in the patient's sample DNA. The cells are viewed with a fluorescent microscope to look for pattern of probes. For example, in a single probe assay, if there is a duplication (or amplification) of the region, more than one probe complementary to that region will be able to hybridize, and multiple probe signals will be visualized by microscopy (Fig. 24.5).

The probes employed in FISH assays are designed to be specific to clinically relevant DNA sequences, such as sites of recurrent translocations, or to specifically label chromosomes or chromosomal regions to allow chromosome enumeration. Probes are generally several hundred base pairs in length and

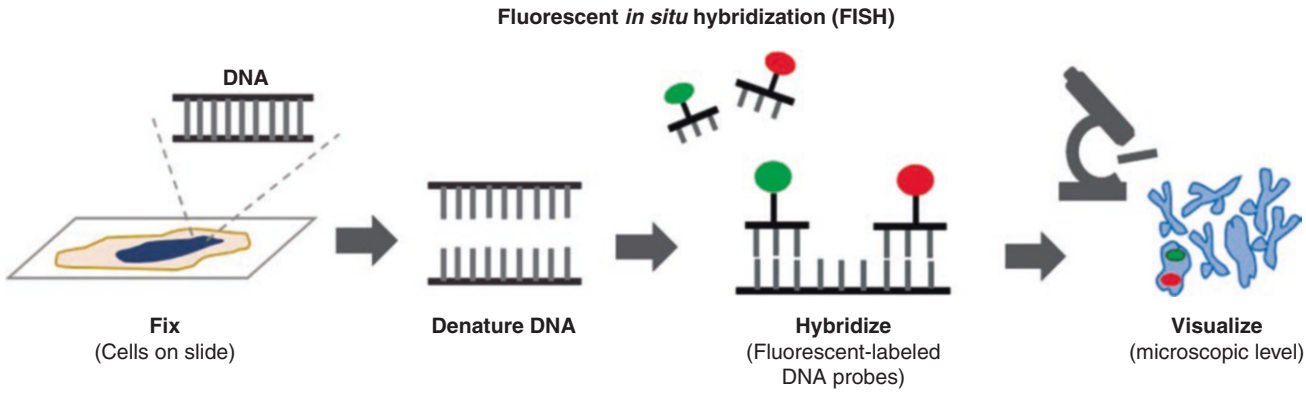

Fig. 24.5 General steps in florescence in situ hybridization (FISH) include (1) fixation of cells onto a glass slide, (2) denaturation of DNA, (3) hybridization of denatured DNA with fluorescent-labeled probes, and (4) visualization of fluorescent signal corresponding to labeled probe using a florescent microscope

hybridize to the DNA region of interest due to their complementary genetic composition. These probes allow for enhanced sensitivity to identify chromosomal abnormalities, including cryptic changes like inv.(16)/t(16;16), whose detection may be unreliable by classical cytogenetics. Moreover, FISH may detect lower frequency alterations because 50–250 or more interphase cells are counted per assay.

Common Applications There are multiple clinically relevant FISH probe types, including break apart, fusion, and chromosome painting probes. This variety allows for a range of assay complexity from simply counting the number of chromosomes to understanding the sites of chromosome breakage and recombination. Some probes are designed to determine whether a breakage event has occurred, as is the cause when a locus (i.e., *MLL* gene) is involved with multiple different partner chromosomes in a given clinical context (i.e., leukemia). As an example of the complexity possible with FISH, the most commonly used probe set for detection of the Philadelphia chromosome is a dual color, dual fusion assay with a red and a green probe to detect involved regions on chromosomes 9 and 22, respectively. These break-apart probes are designed to span the recurrent breakpoint cluster regions (BCRs) so that each colored signal will be split in two if the translocation is present. In this FISH assay, additional complexity is generated by the fusion of the red and green signals on the derivative chromosome to form a yellow signal. As the chromosome 22 probe spans the minor (m)-BCR but not the major (M)-BCR isoform, the assay distinguishes between the two different breakpoints based on the signal output of the assay. The recombination event that produces the Philadelphia chromosome (der(22)) generates a yellow signal due to fusion of one green and one red signal. However, the m-BCR event leaves a small green signal to be detected on the der(9),which is juxtaposed to the remaining red signal to produce a second yellow signal. Conversely, the M-BCR event does not split the green signal, so der(22) appears as a yellow signal, but der(9) appears as a small red signal. In each of these cases, the unaffected chromosomes 9 and 22 give a red and green signal, respectively. Thus, t(9;22) with M-BCR gives one green, two red, and one yellow signal, whereas t(9;22) with m-BCR shows one green, one red, and two yellow. A normal result would be two red and two green signals (diploid for both 9 and 22) (Fig. 24.6).

Array

Array technology allows for evaluation of the expression or genotype of large number of genes or regions of the genome or transcriptome (collection of mRNA molecules). This technique generally involves probes adhered to a solid phase (i.e., multi-well plate or microchip) that are then incubated with a tagged nucleotides (i.e., fluorophore- or chemiluminescence-tagged DNA or RNA) isolated from the patient's sample (DNA or RNA) to allow hybridization. The probe-target hybridizations are then detected and quantified to determine the abundance of nucleic acid sequences. The probe libraries include millions of oligonucleotide designed to give coverage across the genome.

In *array comparative genomic hybridization (aCGH) technique*, the patient sample and a normal reference sample are digested and each labeled with a unique signal (i.e., red and green fluorophore tags) and incubated together on the microchip array. The relative contribution of patient versus normal control is then compared, thus allowing the determination of regions where the patient has either lost or gained genetic material. This information has relevance in detection of small chromosomal deletions and insertions that may be missed by conventional karyotype [12]. aCGH can also be used in assessment of the number of set of chromosomes(ploidy) which is important in risk stratification in ALL. Hyperdiploid and hypodiploid genotypes confer good and poor prognoses, respectively [13, 14].

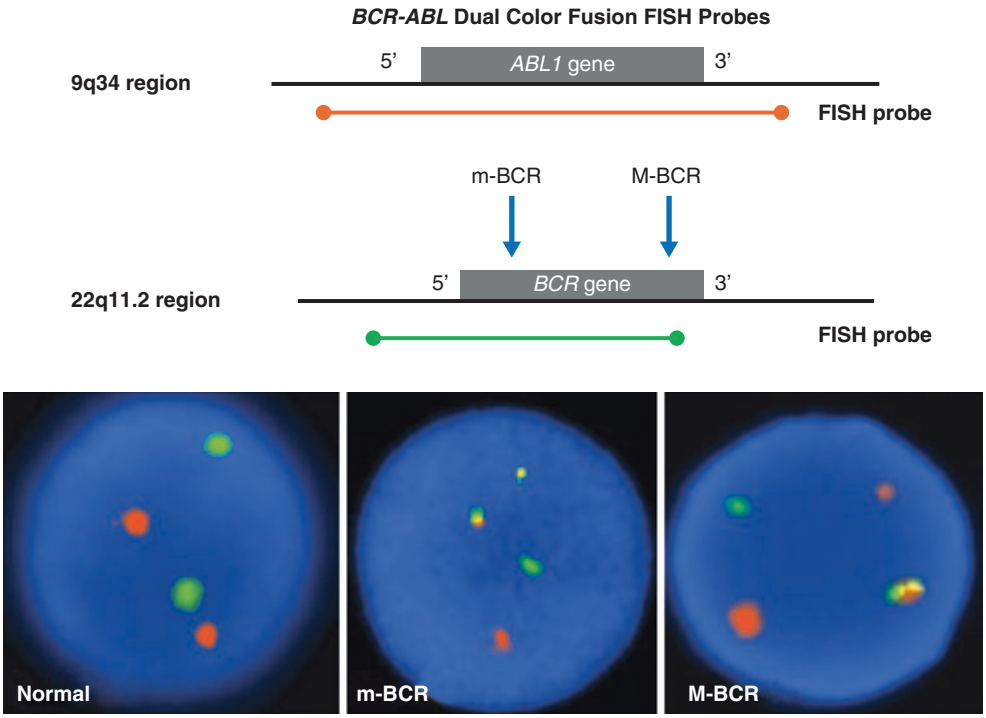

Fig. 24.6 A diagram of BCR-ABL1 FISH probes. Probe diagram adapted from Primo et al. Leukemia 2003. Example FISH results for normal, m-BCR, and M-BCR cases. (FISH images courtesy of Dr. Rose Beck)

In *single-nucleotide polymorphism (SNP) microarray technique*, the probe library includes paired probes fabricated to preferentially bind to one of two SNPs at a locus. With the paired probe arrangement, only the patient sample needs be tested. The sum of fluorescence from the two paired probes gives the "copy number," and the relative ratio between the pair gives the genotype.

Common Applications These array-based techniques allow for genome-wide investigation of copy number at a resolution greater than that of karyotype or FISH and can detect small gains or losses of chromosomal material not detected by karyotype or FISH [12]. While both types of DNA arrays are used clinically, SNP arrays carry the advantage that they can detect copy number neutral loss of heterozygosity or uniparental disomy, which can be an important mechanism of tumor suppressor gene silencing in cancers. Neither technology reliably detects balanced translocations or inversions, as the total zygosity at the involved loci is maintained in these abnormalities (there is no net gain or loss of chromosomal material).

Gene expression profiling (GEP) by *RNA microarray* has largely remained in the realm of research, but clinical applications have emerged. More commonly, research using this technology has produced information that has led to clinical testing for genes that are relevant to prognosis or disease subclasses as in the case for germinal center versus activated B-cell phenotype in diffuse large B-cell lymphoma [15]. While these prognostic indicators were discovered by GEP, they are now most commonly tested for using a surrogate immunohistochemistry algorithm. Clinical utility of GEP has been demonstrated for plasma cell myeloma, where molecular classification can help predict response to and, therefore, guide selection of appropriate therapies [16].

Sequencing

Sequencing technologies have grown dramatically in clinical utility and availability. However, before discussing the technologies themselves, a basic discussion of DNA mutations is necessary. Categories of DNA changes include (1) single-nucleotide variants (point mutations), (2) insertions and deletions, (3) structural variants (i.e., rearrangements or fusions), and (4) copy number changes. Point mutations are alterations of a single base pair. Some point mutations are silent in that they do not alter the encoded amino acid. Others may occur within an intron and have no effect on the protein coding sequence. Point mutations with more obvious effects are missense and nonsense mutations. Missense mutations change the encoded amino acid. Still, the aberrant amino acid may or may not have an effect on the function of the resultant protein. Nonsense mutations, on the other hand, introduce a premature stop codon and are more likely to have a significant effect on protein functionality. Insertions and deletions (indel) sometimes can occur in multiples of three, which will merely add or subtract the number of encoded amino acids. Alternatively, an indel can cause a frameshift with more dramatic effects on protein coding sequence, including ablating or adding a stop codon (Table 24.2).

Table 24.2 Types of and abbreviations used in denoting DNA mutations

				genomic (g.)	coding (c.)	protein (p.)
			Reference sequence(s):	CATGTTAACATGATA	cATGTTAACATGATA	Met-Leu-Thr-Stop (MLT*)
>	Substitution	c.7A>T	p.T3S	CATGTTATCATGATA	cATGTTATCATGATA	Met-Leu-Ser-Stop (MLS*)
fs	Frameshift (change coding frame)	c.7delA	p.T3Hfs	CATGTTA_CATGATA	cATGTTA_CATGATA	Met-Leu-His-Glu (MLHE)
del	Deletion	c.7delA	p.T3Hfs	CATGTTA_CATGATA	cATGTTA_CATGATA	Met-Leu-His-Glu (MLHE)
_	Range (of mutation)	c.7_9del	p.T3del	CATGTTA___TGATA	cATGTTA___TGATA	Met-Leu-Stop (ML*)
dup	Duplication	c.5dupT	p.L2Ffs	CATGTTTAACATGATA	cATGTTTAACATGAC	Met-Phe-Asn-Met (MFNM)
delins	Deletion and Insertion	c.7_8delinsTT	p.L2*	CATGTTATTATGATA	cATGTTATTAATGATA	Met-Leu-Leu-Met-Ile (MLLMI)
ins	Insertion	c.4_5insAA	p.L2*	CATGTAATAACATGATA	cATGTAATAACATGATA	Met-Stop (M*)
inv	Inversion	c.8_11inv	p.*4H	CATGTTAACTCATTA	cATGTTAACTCATTA	Met-Leu-Thr-His (MLTH)
- / +	5' or 3' of coding region	c.1-1C>G		GATGTTAACATGATA	cATGTTAACATGATA	Met-Leu-Thr-Stop (MLT*)

Significant research has allowed the creation of databases that catalog mutations in many disease-associated genes. Variants in a gene are categorized based on the deleterious effect of the alteration. In some cases, the mutation is known to cause disease, but in others, the effect is uncertain or has been shown in epidemiologic or basic research settings not to be disease causing. For that reason, standards have been developed with the goal to annotate each variant with the most up-to-date information available [17, 18]. The variants can be classified as pathogenic, likely pathogenic, uncertain significance (VUS), likely benign, and benign. Factors used to determine these classifications include population, functional, predictive/computational, segregation, allelic, and shared clinical data. As sequencing assays are ordered more frequently in clinical practice, the understanding of this nomenclature is essential. A physician should not overreact to the presence of a variant but take a step back and consider the nature of the specific change (i.e., pathogenic vs. benign). For example, a likely benign variant in a well-known oncogene like c-KIT should not be interpreted as a reason to give the patient a tyrosine kinase inhibitor (TKI) to target this kinase. Conversely, the presence of a pathogenic variant in this kinase in a leukemia patient who has failed conventional therapy may provide rationale to consider enrollment in a clinical trial of or use of a TKI.

Sanger Sequencing

Since its original conception in the 1970s, advances in technology have made DNA sequencing both more rapid and affordable. The workhorse technology employed by the Human Genome Project and still in use in clinical practice today is called Sanger sequencing, named after its developer.

Sanger sequencing technique relies on enzymatic incorporation (by DNA polymerase) of chain terminating dideoxynucleotides (ddNTPs) in a PCR-based assay. Amplified DNA of the region of interest is used as a template for competitive incorporation of dNTPs and labeled ddNTPs. Each of the different ddNTPs (ddATP, ddGTP, ddCTP, ddTTP) carries a unique label. Because the ddNTPs prevent further elongation, the reaction produces PCR products that vary in length corresponding to the terminal nucleotide (ddNTP). The PCR products can be resolved by size and the sequence determined from their associated ddNTP label (Fig. 24.7).

Common Applications Sanger sequencing has the advantage of being a robust, accurate, and relatively inexpensive assay. These reasons make it the "gold standard" of sequencing technologies. For a defined target gene, an assay can be developed to detect a number of pathogenic changes, whereas many PCR-based assays are only capable of determining the presence or absence of one or a small number of defined changes. One drawback of the Sanger method is its sensitivity in detection. Sanger sequencing has an analytical sensitivity of detection of 15–20% variant allele frequency (VAF); it can only reliably identify the mutation if it is present in 1 in 5 DNA molecules in the sample. As a comparison, allele-specific PCR can have an analytical sensitivity of detection of 0.1% (detect a mutation present in 1 in 1000 molecules in the sample). As such Sanger sequencing may fail to detect sub-clonal mutations or mutations in a sample diluted by normal tissue. Furthermore, the Sanger method cannot be multiplexed, that is, each reaction is limited to analyzing a single gene (or region of interest) from a single patient sample (Table 24.3).

Next-Generation Sequencing (NGS)

Newer sequencing technologies such as *next-generation sequencing* (NGS, also called deep sequencing, high-throughput sequencing, or massively parallel sequencing) have emerged that allow for the simultaneous analysis of a panel of genes or even the whole genome. The key difference in NGS from Sanger sequencing is that after the incorporation of a nucleotide that halts synthesis, in NGS, there is a reversal of this "termination" that allows sequencing to continue. As such this method is sometimes referred to as "sequencing by synthesis" because the sequence is determined as the complementary strand is built based on the sequence of the template.

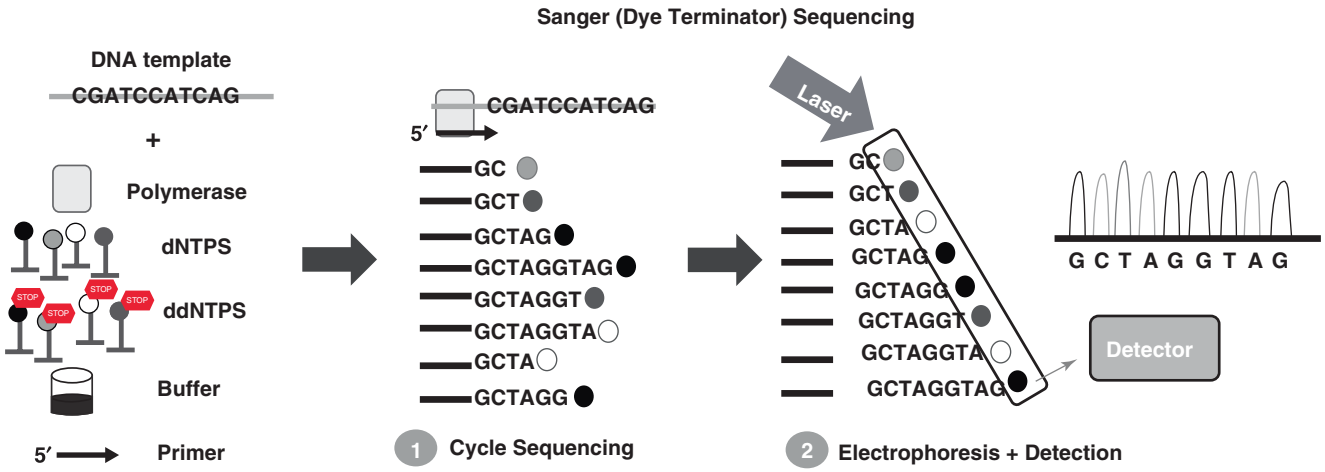

Fig. 24.7 Components of Sanger (dideoxy chain termination) sequencing include a DNA template, a mixture of deoxynucleotide triphosphates (dNTPs) and chain-terminating, fluorescently labeled dideoxynucleotide triphosphates (ddNTPs), a DNA primer, DNA polymerase, and buffer. In step 1, a specific DNA template is used for unidirectional PCR using one primer and mixture of dNTPs and ddNTPS. The random insertion of ddNTPS leads to chain termination (inhibition of further polymerase extension). This produces a range of PCR products of various sizes which are terminated at every possible base over the course of many random insertion reactions. In step 2, the range of products is separated based on the size by capillary electrophoresis; then the fluorescence generated by the terminal fluorochrome-nucleotide is detected following laser excitation. A software then converts the signal type and intensity to normalized peaks that make up an electropherogram for base-calling

Table 24.3 Sensitivity of different methodologies

Technique	Sensitivity of detection
Sanger sequencing	15–20%
Next-generation sequencing	2–5%
Quantitative PCR (qPCR)	1%
Allele-specific PCR (or ARMS)	0.1%
Digital PCR	0.01%

NGS Technique Several companies make NGS platforms with varying methodologies. However, the basic steps of the two most commonly used methods are outlined in Fig. 24.8. First, a sample library must be generated, either using amplification- or capture-based approaches. This process generates target DNA composed of fragments ranging from less than 100 to several hundred base pairs in length. These fragments are then ligated to an adapter that serves as a barcode, allowing for sequencing of multiple samples in a single sequencing run. The bar-coded DNA undergoes clonal amplification on a solid surface (i.e., flow cell or bead) and then sequencing by methods that differ across NGS platforms (i.e., fluorescence detection or pH changes). The data output includes hundreds of millions of base pair reads from the input DNA fragments. Sophisticated software then allows the alignment of the fragments to a reference genome to produce the patient sequence. Finally, calling of variants occurs based on a threshold of certainty for detected deviations from the reference genome. The level of sensitivity of this assay is contingent on the depth of coverage (number of unique reads) at that locus.

Common Applications While NGS is capable of sequencing an entire whole genome or exome, most commonly, a targeted selection of genes of clinical interest is sequenced. In the context of hematology, this technology is often used to investigate the presence of disease-defining or disease-associated mutations that may help clarify a diagnosis or offer information about prognosis or possible targeted therapy. Most commonly, these sequencing panels are used for myeloid malignancies. Analogous panels for lymphoid neoplasms are becoming more common. Outside of neoplastic hematology, gene sequencing panels are used for bone marrow failure syndromes and chromosome breakage syndromes where mutation in any of a number of different genes may produce a similar disease phenotype.

Sequencing methods do not require prior knowledge of the DNA sequence or variant, just the flanking regions. Genes with characteristic "hot spot" (known) mutations, as occur in *JAK2* or *MYD88* genes are more easily amenable to PCR-based techniques. However, truncation mutations in tumor suppressors often occur in a broad distribution along that gene. It is this category of genes with scattered mutations that are best characterized by sequencing technologies due to the unbiased nature of the technology. Sequencing assays are designed to capture the loci where the majority of mutations occur. Based on assay design, NGS technologies can be used to detect all four types of DNA alterations: (1) single-nucleotide variants (SNV), (2) insertions and deletions (Indel), (3) structural variants (SV), and (4) copy number changes (CNV) (Table 24.4).

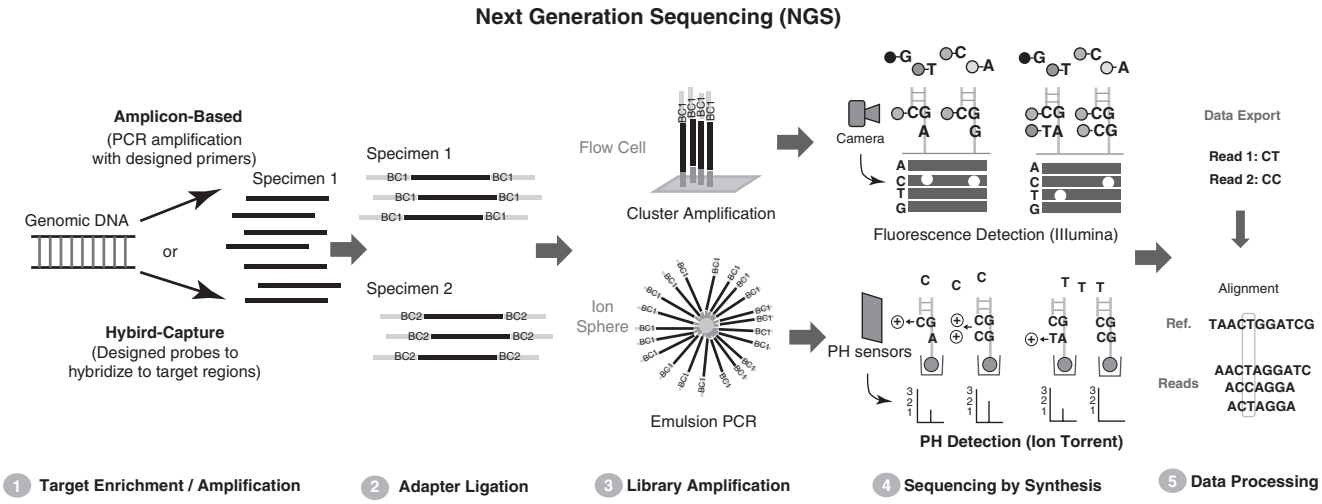

Fig. 24.8 There are several methodologies that can be used for next-generation sequencing. The basic conventional steps of the two most commonly used methods for targeted NGS are outlined. (1) First, the target regions of interest are enriched using either an amplification- or capture-based approach. (2) Next "enriched" DNA fragments are ligated to an adapter-barcode sequence that consists of a barcode (unique sample identifier sequence) and adapter (allows binding to a solid state) for further amplification and sequencing. (3) The collection of adapter-barcoded DNA sequences is referred to as a "library." This DNA library (which usually consists of multiple patient samples in a single run) undergoes clonal amplification on a solid surface. Examples of a solid surface include a flow cell for cluster amplification or a bead for emulsion PCR amplification. (4) Next, each unique DNA fragment that had undergone clonal amplification is sequenced during synthesis. Two common methods of reading the sequence are by detection of an incorporated fluorescently labeled nucleotide or by pH changes that arise from incorporation of nucleotides during synthesis. This reading of the sequence is performed as the sequence synthesis is halted either by fluorophore-labeled nucleotide or the lack of complementary nucleotide for extension. Then this stoppage is reversed chemically or by the availability of appropriate nucleotides. As such, NGS is often referred to as "sequencing by synthesis" because the sequence is determined as the complementary strand is built based on the sequence of the template. (5) Software converts the fluorescence or pH changes into a base call. This outputted digital sequence files that contain millions of base pair reads from the input DNA fragments are then aligned to a reference genome by sophisticated software to produce the patient sequence

Table 24.4 Types of molecular tests and variants detected

Molecular methodology	SNV	Indel	SV	CNV
ASO-PCR (or ARMS)	x			
MLPA	x			x
Array		x		x
FISH			x	x
Sanger sequencing	x	x		
NGS	x	x	x	x

Conclusions

Conventional morphologic, phenotypic, and cytogenetic analysis is widely used in the clinical care of patients with hematologic disorders. Although these methods serve as the diagnostic foundation, the use of newer molecular techniques has provided new insights creating more complete diagnosis in clinical hematology. FISH, PCR, and NGS techniques have been transformative for detecting disease-defining or disease-associated fusions. Sequencing technologies have led to discovery of many important biomarkers, such as CALR mutations in myeloproliferative disease. More sensitive techniques such as qPCR, ARMS, and digital PCR have paved the way for better monitoring of residual disease and response to therapy. The continued improvement of next-generation sequencing technologies may 1 day make it a one-stop source for genetic information about a patient. Moreover, with advances in bioinformatics, the integration of complex clinical datasets and genetic test results may help to better derive optimal and precise treatment plans. Finally, at present, it is essential to understand these techniques since in the broad field of hematology, these analyses are essential for diagnosis and management.

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Chronic Myeloid Leukemia (CML)

25

Thomas G. Knight, Michael R. Grunwald, and Edward A. Copelan

Definition

Chronic myeloid leukemia (CML) is a clonal myeloproliferative expansion of transformed, primitive hematopoietic progenitor cells [1].

Pathophysiology

CML is a result of a t(9;22) which is a reciprocal chromosomal translocation between the ABL1 proto-oncogene on chromosome 9 and the BCR gene on chromosome 22 [2]. ABL proteins are nonreceptor tyrosine kinases that have important roles in signal transduction and the regulation of cell growth [1]. However, this is normally regulated by an autoinhibitory mechanism. The fusion with BCR disrupts the internal control mechanism that keeps c-Abl in an inactive form [3]. The resulting product (BCR-ABL1) is a constitutively active tyrosine kinase leading to uncontrolled proliferation of myeloid, monocytic, erythroid, megakaryocytic, B-lymphoid, and occasionally T-lymphoid cell lineages [1]. BCR-ABL1 accomplishes this propagation by inhibiting apoptosis and stimulating cell cycle entry of hematopoietic cell lines even in the absence of growth factors [4]. Of note, there are several variations of the BCR-ABL1 fusion protein generated, depending upon the location of the breakpoint on chromosome 22. The most common variant derives from a breakpoint in exon b2 or b3 in BCR, fused to ABL1 at exon a2 which leads to a 210 kda protein commonly referred to as the p210 BCR-ABL1 protein. An alternative BCR exon splice at e19 can lead to a p230 BCR-ABL1 protein. Finally, a BCR splice at e1 can lead to a p190 BCR-ABL1 protein which occurs in a very small number of CML patients but more frequently in Ph-positive acute lymphoblastic leukemia (ALL) [1]. These mutations also have different clinical activities with the shorter protein (p190) leading to more aggressive cell behavior than the longer proteins [5].

Diagnosis

(a) *Clinical Presentation*
Patients typically present with an elevated WBC count with large numbers of immature cells, increased basophils, and an enlarged spleen [6]. Symptoms at presentation can include fatigue, bleeding, weight loss, infection, early satiety, abdominal pain, and sweating at rest and with exercise [7].

(b) *Differential Diagnosis*
As the presentation is fairly nonspecific, the differential diagnosis is quite broad. In terms of nonmalignant causes, leukemoid reactions can present with a CML-type picture, and there is often a clear infectious cause for the reaction. However, in some cases, metastatic cancer can also lead to a leukemoid reaction. Other malignant hematologic causes can be separated into Philadelphia chromosome-negative and Philadelphia chromosome-positive malignancies. The differential diagnosis for BCR-ABL1-negative hematologic neoplasms includes atypical CML, chronic myelomonocytic leukemia (CMML), chronic neutrophilic leukemia (CNL), and myelodysplastic syndrome/myeloproliferative neoplasm unclassifiable (MDS/MPN, U). Of note, "atypical CML" has been given the historical moniker "CML-like syndrome," because both diseases exhibit bone marrows with hyperplastic myeloid hyperplasia and peripheral blood leukocytosis characterized by a spectrum of myeloid immaturity. However, on morphologic grounds, this is where the similarity ends. Unlike BCR-ABL1-positive CML, atypical CML is characterized by prominent dysplastic granulopoiesis (e.g., the acquired Pelger-Huët anomaly; nuclear abnormalities including hypersegmentation, nuclear projections, and

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abnormally clumped nuclear chromatin; and abnormalities of cytoplasmic granules, such as hypogranularity), and in some cases, multilineage dysplasia may be observed [8]. Philadelphia chromosome-positive malignancies mainly consist of acute lymphoblastic leukemia (ALL).

(c) *Workup*
All patients with a suspected new diagnosis of CML should have a CBC with differential and peripheral smear examined. FISH or PCR for BCR-ABL1 is performed on the peripheral blood to confirm the diagnosis. However, all patients also will require a bone marrow aspirate and biopsy with conventional cytogenetics. This procedure allows for proper staging (chronic, accelerated, or blastic phase) of the disease and identifies marrow fibrosis and additional cytogenetic abnormalities and can therefore affect treatment decisions.

(d) *Staging*
CML can be classified as being in chronic, accelerated, or blastic phase. WHO criteria for accelerated and blast phase are as follows [9]:

(i) *CML accelerated phase (diagnose if one more of the following is present)*
1. Blasts comprising 10–19% of peripheral blood white cells or bone marrow cells
2. Peripheral blood basophils of at least 20%
3. Persistent thrombocytopenia ($<100\times10^9$/L) unrelated to therapy or persistent thrombocytosis ($>1000\times10^9$/L) unresponsive to therapy
4. Increasing spleen size and increasing WBC count unresponsive to therapy
5. Cytogenetic evidence of clonal evolution (i.e., the appearance of an additional genetic abnormality that was not present in the initial specimen at the time of diagnosis of chronic phase CML).
6. Megakaryocytic proliferation in sizable sheets and clusters, associated with marked reticulin or collagen fibrosis, and/or severe granulocytic dysplasia, should be considered as suggestive of CML-AP. These findings have not yet been validated in large clinical studies; however, it is not yet clear if they should represent independent criteria for accelerated phase. These features often occur simultaneously with one or more of the other features listed.

(ii) *CML blast phase (diagnose if one more of the following is present)*
1. Blasts comprising 20% or more of peripheral blood white cells or bone marrow cells
2. Extramedullary blast proliferation
3. Large foci or clusters of blasts in bone marrow biopsy

Prognosis

The development of tyrosine kinase inhibitors (TKI) has greatly extended life expectancy in patients with CML, which is now approaching that of the general population. It is estimated that patients of all ages diagnosed after 2011 will, on average, lose less than 3 life-years as a result of CML [10]. There are several scoring systems available to aid in determining prognosis. They are summarized below. However, it must be noted that the Sokal score and the Euro score were developed prior to the development of TKI therapy, and the EUTOS score was developed using imatinib but has not been validated with the newer TKIs, which are more potent than imatinib.

(a) *Sokal Score* [11]
(i) Developed to aid in prognosis and predict response to treatment with conventional chemotherapy (e.g., busulfan)
(ii) Variables include age (years), spleen size below costal margin (cm), platelet count (10^9/L), and blast percentage
(iii) Was able to identify a lower-risk group of patients with a 2-year survival of 90% and median survival of 5 years; an intermediate risk group; and a high-risk group with a 2-year survival of 65%, subsequent mortality rate of about 35% per year, and median survival of 2.5 years

(b) *Euro Score* [12]
(i) Developed to aid in prognosis and predict response to treatment with alpha-interferon.
(ii) Variables include age, spleen size, blast count, platelet count, eosinophil count, and basophil count.
(iii) Was able to identify three distinct risk groups with median survival times of 98 months, 65 months, and 42 months.

(c) *EUTOS Score* [13]
(i) Developed to aid in prognosis and predict response to treatment with imatinib
(ii) Variables are basophil percentage and spleen size
(iii) Was able to identify high-risk and low-risk groups of patients, with progression-free 5-year survival significantly better in the low-risk group compared to the high-risk group (90% vs 82%, $P = 0.006$)

Treatment of CML in Chronic Phase

(a) *Selection of TKI*

Once the diagnosis is confirmed, therapy with a TKI should be started. There are four drugs approved for first-line treat-

ment of CML in chronic phase—the first-generation TKI, imatinib, and three second-generation TKIs, dasatinib, bosutinib, and nilotinib. The selection of which drug to use first remains controversial and is patient-specific. It has been our general practice to use second-generation TKIs in the first line, but there are several factors that must be considered. Specific factors include the patient's comorbidities, the side effect profile, desired speed/depth of response, cost, and patient preference. One might avoid nilotinib in patients with a history of peripheral arterial disease, diabetes mellitus, or gastrointestinal disease, and one might avoid dasatinib in patients with a history of pleural effusions. In terms of speed/depth of response, the major studies evaluating the second-generation TKIs (DASISION, ENESTnd, ENESTchina) reveal that these drugs obtain faster and deeper responses; nevertheless, the speed and depth of response have not yet translated into increased overall survival [14–16]. In terms of cost, TKIs can vary widely depending on location and insurance status. As of 2012, in the United States, all five TKIs approved for CML have drug acquisition costs of $92,000–$138,000 annually [17]. However, a generic form of imatinib has now entered the US market as of February 2016. Although initially priced at approximately $140,000 per year, this price will be significantly lower in the coming years [18]. At the same time, it is unlikely that there will be manufacturer's assistance programs available for generic imatinib, so affordability may still be a concern. Finally, patient preference must be considered, since adherence is the single most important factor in efficacy of TKI therapy. Scheduling (once daily vs twice daily) and/or the ability to take food with the medication may be important factors for the patient.

(b) *Monitoring Therapy*

(i) *Response Criteria* [19]

1. *Complete Hematologic Response (CHR)*: White blood cell count <10,000/μL with no immature granulocytes and <5% basophils on differential, platelet count <450,000/μL, and spleen not palpable.
2. *Cytogenetic Response (CyR)*: Assessed by chromosome banding analysis (CBA) of marrow cell metaphases with at least 20 metaphases analyzed. Cytogenetic response is classified according to the percent Philadelphia chromosome positive cells into no response (>95%), minimal (66–95%), minor (36–65%), major (1–35%), and complete (no Philadelphia chromosome positive cells).
3. *Molecular Response*: Assessed according to the International Scale (IS) as the ratio of BCR-ABL1 transcripts to ABL1 transcripts or other internationally recognized control transcripts, and it is expressed and reported as BCR-ABL1% on a log scale, where 10%, 1%, 0.1%, 0.01%, 0.0032%, and 0.001% correspond to a decrease of 1, 2, 3, 4, 4.5, and 5 logs. The IS defines 100% as the median pretreatment baseline level of BCR-ABL1 RNA in early chronic phase CML (as determined in the IRIS trial). A BCR-ABL1 expression of ≤0.1% corresponds to major molecular response (MMR). $MR^{4.0}$ = either (i) detectable disease with <0.01% BCR-ABL1 IS or (ii) undetectable disease in cDNA with >10,000 ABL1 transcripts; $MR^{4.5}$ = either (i) detectable disease with <0.0032% BCR-ABL1 IS or (ii) undetectable disease in cDNA with >32,000 ABL1 transcripts in the same volume of cDNA used to test for BCR-ABL1.

(ii) *Monitoring Schedule*

At diagnosis, all patients should undergo a bone marrow aspirate and biopsy to perform chromosome banding analysis of marrow cell metaphases and PCR of BCR-ABL1 in the peripheral blood. Following initiation of TKI therapy, patients should be closely monitored with physical exams, complete blood counts with differentials, and metabolic profiles as indicated every 1–2 weeks to assess for side effects from therapy and hematologic response. If the patient achieves a complete hematologic response and is having no significant side effects from his or her medication, then testing and visits can be spaced out per ELN guidelines for assessing response. Optimal response criteria, warning signs, and treatment failure have been defined by the ELN and are detailed in Table 25.1. While some centers now monitor response exclusively by peripheral blood PCR and do not repeat marrow cytogenetics unless there is failure to achieve MMR within 12 months, both should be obtained until complete cytogenetic response and MMR are obtained. Patients who achieve MMR should then be monitored every 3–6 months with a complete blood count with differential and PCR of BCR-ABL1 in the peripheral blood.

(iii) *Stopping TKI Therapy*

There is emerging data that TKI therapy can be safely discontinued in a subset of patients who have achieved deep remission (≤ MR^4) [20–22]. Guidelines are evolving, but there is general emphasis on ensuring patient safety by restricting discontinuation to CML-CP patients with no prior history of advanced disease, prior TKI therapy of 5 years, stable deep molecular remission ((≤ MR^4) of 2 years duration, and access to frequent monitoring with a sensitive (≤ MR4.5) qRT-PCR standardized

Table 25.1 ELN Definition of the response to TKIs as first-line treatment [19]

	Optimal	Warning	Failure
Baseline	NA	High risk Or CCA/Ph+, major route	NA
3 months	BCR-ABL1 ≤10% and/or Ph+ ≤35%	BCR-ABL1 >10% and/or Ph+ 36-95%	Non-CHR and/or Ph+ >95%
6 months	BCR-ABL1 <1% and/or Ph+ 0	BCR-ABL1 1–10% and/or Ph+ 1–35%	BCR-ABL1 >10% and/or Ph+ >35%
12 months	BCR-ABL1 ≤0.1%	BCR-ABL1 >0.1–1%	BCR-ABL1 >1% and/or Ph+ >0
Then and at any time	BCR-ABL1 ≤0.1%	CCA/Ph– (–7, or 7q–)	Loss of CHR
			Loss of CCyR
			Loss of MMR
			BCR-ABL1 mutations
			CCA/Ph+

CCA/Ph+ clonal chromosomal abnormalities in Ph+ cells, *CHR* complete hematologic response (Platelets $<450\times10^9$/L, and white cells $<10\times10^9$/L, and no circulating immature myeloid cells, and <5% basophils on differential, and no palpable splenomegaly); CCyR = No Ph+ cells*; fewer than 1 out of 200 nuclei BCR-ABL1–positive by FISH; MMR = BCR-ABL1 ≤0.1%

to the International Scale with a rapid turnaround time of 2–4 weeks [22]. Examples of this shift in thinking include the NCCN now providing recommendations on TKI cessation and the US Food and Drug Administration updating the product label for nilotinib to include information for providers about how to discontinue the drug in certain patients [23, 24]. Studies do show though an approximately 50% relapse rate which usually occur within the 1st year. However, the vast majority (but not all) of these patients will be able to regain the prior depth of MR upon rechallenging with the same TKI [25]. These studies also have contributed to more second-generation TKIs being used in front line with these agents showing faster and deeper responses than imatinib and thus potentially allow more patients to meet criteria for a trial of stopping therapy [14–16].

(c) *Failure of Initial Therapy*

(i) *Drug Intolerance*

As detailed below, each of the TKIs has short-term and long-term side effects that can necessitate stopping the medication. In the event of mild to moderate adverse effects, one can attempt a short break from therapy and rechallenge with the same medication following resolution of symptoms. However, in the case of grade III/IV toxicities or severe patient intolerance, it is advisable to stop the TKI and switch to another TKI with a different side effect profile.

(ii) *Resistance*

This refers to both primary resistance, in which patients are unable to achieve proper milestones from the beginning of therapy, and secondary resistance, in which patients exhibit a loss of MMR despite TKI therapy. Regardless, the first step when there is concern for resistance to TKI therapy is to have a detailed discussion with the patient about adherence, since lack of adherence is the best predictive factor for resistance. In one study, an electronic device was fitted in the cap of a normal-looking medication bottle that then automatically recorded each time the bottle was opened. Investigators were able to demonstrate that adherence to therapy is associated with molecular response at 18 months and was the most important factor influencing the depth of response in patients achieving CCyR. No CMRs were observed when adherence was ≤90%, and no MMRs were observed when adherence was ≤80% [26]. If resistance is identified, then a bone marrow biopsy should be performed with cytogenetic testing. There should also be a mutational analysis of BCR-ABL1 performed, since the presence of resistance mutations can guide the choice of next therapy. If the patient has progressed to accelerated or blast phase, then the provider should treat per guidelines for advanced disease.

(iii) *BCR-ABL1 Mutations*

The development of mutations within the kinase domain of *BCR-ABL1* represents the most frequent mechanism of resistance to TKI therapy in CML, being detected in 40% to 90% of patients who are resistant to imatinib [27]. This most often occurs at the binding site for the TKI. Therefore, this will create different patterns of resistance/effectiveness based on where the TKIs bind. Analysis for mutations in BCR-ABL1 is recommended for all patients who lose response while on TKI therapy [28, 29]. Common mutations and switching strategies are below:

1. *T315I*: Resistant to all TKIs except ponatinib. There is some activity with omacetaxine. Patients with this mutation should be considered for allogeneic hematopoietic cell transplant.
2. *V299L*, *T315A*, *and F317L/V/I/C*: Resistant to dasatanib, may be sensitive to nilotinib.
3. *E255K/V*, *Y253H*, *and F359V/C/I*: Resistant to nilotinib and imatinib but may be sensitive to dasatanib.

(iv) *Treatment After Initial TKI Resistance*

1. *Change in TKI Therapy*: This situation should include a change in dose of the TKI or use of an alternative TKI. Switching of TKI should be based on both mutational findings and side effect profile.

2. *Allogeneic Hematopoietic Cell Transplantation (HCT)* Prior to the advent of TKIs, HCT was the definitive therapy for CML, and most of the data about efficacy are from this time period. For example, one study of 117 patients with CML who underwent transplant between 1978 and 1982 showed 3-year survivals of 63%, 56%, and 16% for patients who underwent transplantation in the chronic, accelerated, and blast phase, respectively [30]. However, the outcome of allogeneic HCT for CML has steadily improved over time. Probabilities of 2-year survival of patients who underwent transplantation in the first chronic phase during the 1980s, 1990s, and between years 2000 and 2003 were 59%, 66%, and 70%, respectively. This was thought to be secondary to a reduction in transplant-related mortality from 41% to 30% in all patients and from 31% to 17% in low-risk patients [31]. Following the development of TKIs, allogeneic HCT has been reserved for patients who are resistant or intolerant to at least two TKIs due to the efficacy and safety of these medications.

Treatment of CML in Accelerated Phase (AP)/ Blast Crisis(BC)

(a) *Prevalence/General Principles*
Treatment of AP and BC CML remains challenging even in the age of TKIs. Although there has been a major improvement in prevention of progression to these stages (progression of CP-CML to AP and/or BC CML has been reduced to 1–1.5% per year compared to more than 20% per year in the pre-imatinib era), some patients will either present with advanced disease or progress after developing resistance to TKI therapy [32]. Generally, patients are first given induction therapy followed by allogeneic HCT, due to data showing that the single most important prognostic factor for survival following allogeneic HCT for CML is the pretransplant disease phase [32, 33]. Therefore, every effort is made to "downstage" patients prior to HCT. Finally, for all patients who are resistant or have progressed despite TKI therapy, it is recommended that a full analysis for mutations in BCR-ABL1 is performed.

(b) *Accelerated Phase*
(i) *General Principles*
Induction is primarily accomplished with TKI therapy. Selection of TKI is based on patient and disease factors.
(ii) *Induction (AP at presentation)*
For patients who are treatment naïve, the general recommendation is to use second- generation TKIs as studies of these agents showing faster and deeper responses than imatinib [14–16]. However, one can consider using imatinib at the higher dose of 600 mg daily if there is an access or cost issue.
(iii) *Induction (AP following treatment)*
If patients progress to AP CML after prior treatment with a TKI, a full analysis for BCR-ABL1 mutations should be performed, and a new TKI should be selected based on the results (see BCR-ABL1 mutations above). If there is not mutational data to guide therapy, the provider can choose a new TKI based on side effect profile and availability. Small studies suggest that the majority of patients will be able to achieve a return to CP or complete remission with TKI therapy alone.
(iv) *HCT*
The majority of patients will achieve a return to CP or complete remission with TKI therapy alone. The current recommendation is to take these patients to HCT at the time of maximal response to therapy. There has not been a randomized trial looking at HCT vs continuing the next TKI. If patients are ineligible for HCT, it is advisable to maintain TKI therapy with continued monitoring.

(c) *Blast Crisis*
(i) *General Principles*
Can present in multiple forms based on clonal origin (myeloid, lymphoid, or undifferentiated). This lineage infidelity suggests that blast crisis in CML represents an arrest of differentiation at an early stage when compared to de novo acute leukemias and thus able to occur along different lineages based on the predominant expression [34]. Myeloid blast phase occurs in 50–60% of cases of BP-CML, whereas lymphoid blast phase is seen in approximately 20–30% of cases; 15–20% of cases have an undifferentiated phenotype [35]. Induction therapy should be selected based on the type of BP.
(ii) *Induction (Lymphoid Blast Crisis)*
Generally treated with TKI plus ALL-type regimen. In one study, patients with lymphoid blastic phase CML were treated with hyperfractionated cyclophosphamide (with hyperfractionated meaning that the total dose is administered in multiple smaller—or "fractionated"—doses to minimize side effects), vincristine, adriamycin, and dexamethasone (HCVAD) plus imatinib or dasatinib. Complete hematological response was achieved in 90% of patients, complete cytogenetic remission in 58%, and complete molecular remission in 25% [35].
(iii) *Induction (Myeloid Blast Crisis)*
Consider TKI plus AML-type chemotherapy. In general, it is recommended to use second-generation TKIs with studies showing faster and deeper

responses than with imatinib [14–16]. However, if the patient has progressed on therapy, a full analysis for mutations in BCR-ABL1 should be performed, and therapy can be chosen based on these results.

(iv) *HCT*
The current recommendation is to take these patients to HCT at the time of maximal response to therapy, but in the era of increasing TKI options, there has not been a randomized trial looking at HCT vs continuing the next TKI. If patients are ineligible for HCT, it is advisable to maintain TKI therapy with continued monitoring.

Medications

(a) *Imatinib*
- (i) *Brand Name*: Gleevec
- (ii) *Classification/MOA*: First-generation tyrosine kinase inhibitor (TKI)
- (iii) *Relevant Studies*: The International Randomized Study of IFN versus STI571 (IRIS) established the superiority of imatinib 400 mg daily compared with IFN-α and low-dose cytarabine (AraC) regarding the rate of hematologic response (HR), cytogenetic response (CgR), and molecular response (MolR), and the study suggested a substantial advantage in progression-free survival (PFS) and overall survival (OS) [36]. At 60 months, the estimated event-free survival (EFS) rate was 83% (95% confidence interval [CI], 79–87), and an estimated 93% of patients (95% CI, 90–96) had not progressed to the accelerated phase or blast crisis. The estimated OS rate at 60 months was 89% (95% CI, 86–92) [37]. Similar results at 5 years were seen in studies from the United Kingdom (EFS of 81.3% and OS of 83.2%) and Germany (EFS of 80% and OS of 94%) [38, 39].
- (iv) *Initial Dosage*: Chronic phase: 400 mg/day. Accelerated phase: 600–800 mg/day
- (v) *Major Side Effects*: Myelosuppression, hepatotoxicity, nausea, edema, fatigue, headaches, muscle cramps, arthralgias, myalgias, diarrhea, and rashes. In some patients, severe rashes can develop with desquamation. Stevens-Johnson syndrome (SJS) has been reported [40].
- (vi) *Reasons to Discontinue*: Progression of disease, grade 3 or 4 myelosuppression, SJS, grade 3 elevations in transaminases (> five times the upper limit of normal), and severe edema.

(b) *Nilotinib*
- (i) *Brand Name*: Tasigna
- (ii) *Classification/MOA*: Second-generation TKI.
- (iii) *Relevant Studies*: In the phase III Evaluating Nilotinib Efficacy and Safety in Clinical Trials-Newly Diagnosed Patients (ENESTnd) study, patients with newly diagnosed chronic phase CML were randomized (stratified by Sokal risk score at baseline) 1:1:1 to receive nilotinib 300 mg twice daily (n = 282), nilotinib 400 mg twice daily (n = 281), or imatinib 400 mg once daily (n = 283). By 5 years, more than half of all patients in each nilotinib arm (300 mg twice daily, 54%; 400 mg twice daily, 52%) achieved a molecular response 4.5 ($MR^{4.5}$; *BCR-ABL1* ≤0.0032% on the International Scale) compared with 31% of patients in the imatinib arm. Rates of EMR and *BCR-ABL1*^{IS} ≤1% at 3 months were higher in the nilotinib arms than in the imatinib arm [15]. A phase III study of nilotinib vs imatinib in Chinese patients with newly diagnosed chronic myeloid leukemia in chronic phase (ENESTchina) also revealed a statistically significant higher rate of major molecular response (MMR; BCR-ABL1 ≤0.1% on the International Scale) at 12 months in the nilotinib arm vs the imatinib arm (52.2% vs 27.8%; P < 0.0001), and MMR) rates remained higher with nilotinib vs imatinib throughout the follow-up period [16].
- (iv) *Initial Dosage*: Chronic phase: 300 mg BID. Accelerated phase or resistant to initial therapy: 400 mg BID
- (v) *Major Side Effects*: Myelosuppression, increased QTc interval on electrocardiogram, severe peripheral artery occlusive disease, rash, pruritus, nausea, fatigue, headache, constipation, diarrhea, vomiting, elevated lipase, hyperglycemia, hypophosphatemia, and elevated bilirubin [41]
- (vi) *Reasons to Discontinue*: Progression of disease, grade 3 or 4 myelosuppression, QTc >500 ms, and development of severe peripheral artery occlusive disease
- (vii) *Black Box Warning*: Avoid drugs known to prolong the QT interval and strong CYP3A4 inhibitors, avoid taking nilotinib with food, use caution in patients with hepatic impairment, and monitor for QT interval prolongation. Abnormalities in potassium and magnesium levels must be corrected prior to drug initiation, and electrocardiograms should be obtained at baseline, after 1 week of treatment, with any dose change, and serially during therapy.

(c) *Dasatinib*
- (i) *Brand Name*: Sprycel
- (ii) *Classification/MOA*: Second-generation TKI
- (iii) *Relevant Studies*: The Dasatinib Versus Imatinib Study in Treatment-Naïve Chronic Myeloid

Leukemia Patients (DASISION) study was a randomized phase III trial comparing the efficacy and safety of dasatinib (100 mg once daily) with imatinib (400 mg once daily) in patients with newly diagnosed CML in chronic phase. Cumulative, 5-year MMR and $MR^{4.5}$ rates were 76% and 42% for dasatinib and 64% and 33% for imatinib, respectively ($P = 0.0022$ and $P = 0.0251$, respectively). Estimated 5-year OS was 91% for dasatinib and 90% for imatinib (HR, 1.01; 95% CI, 0.58–1.73). Estimated 5-year PFS was 85% for dasatinib and 86% for imatinib (HR, 1.06; 95% CI, 0.68–1.66) [14]. Similar results were found in a phase II study comparing dasatinib 100 mg to imatinib 400 mg in newly diagnosed chronic phase CML. The proportion of patients achieving a complete cytogenetic remission rate was superior with dasatinib (84% vs 69%), as was the 12-month molecular response by the proportions of patients achieving>3-log,>4-log, and >4.5-log reduction in BCR-ABL1 transcript levels [42].

(iv) *Initial Dosage*: Chronic phase: 100 mg daily. Accelerated phase 140 mg daily.

(v) *Major Side Effects*: Myelosuppression, fluid retention (can be severe; includes pleural effusions), pulmonary arterial hypertension, QT prolongation, diarrhea, headache, musculoskeletal pain, and rash.

(vi) *Reasons to Discontinue*: Pleural effusions, Progression of disease, grade 3 or 4 myelosuppression, QTc >500 ms

(d) *Bosutinib*

(i) *Brand Name*: Bosulif

(ii) *Classification/MOA*: Second-generation TKI

(iii) *Relevant Studies*: Initially approved based on the phase I/II study where bosutinib 500 mg was given to patients with chronic phase imatinib-resistant or imatinib-intolerant CML. After a median follow-up of 24.2 months, 86% of patients achieved complete hematologic remission, 53% had a major cytogenetic response (41% had a complete cytogenetic response), and 64% of those achieving complete cytogenetic response had a major molecular response. At 2 years, progression-free survival was 79%; overall survival at 2 years was 92%. Responses were seen across BCR-ABL1 mutants, except T315I [43]. The efficacy and safety of bosutinib 500 mg/d ($n = 250$) versus imatinib 400 mg/d ($n = 252$) were first assessed in newly diagnosed chronic phase CML in the Bosutinib Efficacy and Safety in Newly Diagnosed CML trial (BELA). Cumulative complete cytogenetic response (CCyR) rates by 24 months were similar (bosutinib, 79%; imatinib, 80%); cumulative MMR rates were 59% for bosutinib and 49% for imatinib [44]. Despite an improvement in MMR rate at 12 months, shorter time to response, and a lower rate of transformation to accelerated phase (AP) or blast phase (BP) with bosutinib, the study's primary objective (superior rate of CCyR at 12 months) was not met. This lead to the phase III BFORE trial (Bosutinib in First-Line Chronic Myelogenous Leukemia Treatment) where 536 patients with newly diagnosed chronic-phase CML were randomly assigned 1:1 to receive 400 mg of bosutinib once daily ($n = 268$) or imatinib ($n = 268$). The major molecular response (MMR) rate at 12 months (primary end point) was significantly higher with bosutinib versus imatinib (47.2% vs 36.9%, respectively, $P = 0.02$), as was complete cytogenetic response (CCyR) rate by 12 months (77.2% vs 66.4%, respectively, $P = 0.0075$). Cumulative incidence was favorable with bosutinib (MMR: hazard ratio, 1.34; $P = 0.0173$; CCyR: hazard ratio, 1.38; $P < 0.001$), with earlier response times [45]. This lead to FDA approval for bosutinib in newly diagnosed chronic phase CML patients.

(iv) *Initial Dosage*: Chronic phase: 400 mg once daily. Accelerated phase or resistant to initial therapy: 500 mg daily. Can consider dose escalation to 600 mg daily in patients who do not reach CHR by week 8 or a CCyR by week 12.

(v) *Major Side Effects*: Diarrhea (68% of patients in the phase III trial), vomiting (32% of patients in the phase III trial), myelosuppression, abdominal pain, elevated liver enzymes, pancreatitis, and edema.

(vi) *Reasons to Discontinue*: GI side effects, progression of disease, grade 3 or 4 myelosuppression, grade 3 elevations in transaminases (> five times the upper limit of normal), and pancreatitis.

(e) *Ponatinib*

(i) *Brand Name*: Iclusig

(ii) *Classification/MOA*: Third-generation TKI

(iii) *Relevant Studies*: Initially FDA approved based on the phase II PACE trial which enrolled 449 patients, 203 of whom had chronic-phase CML and resistance or intolerance to dasatinib or nilotinib and 64 of whom had chronic-phase CML characterized by the T315I mutation. Patients received 45 mg of ponatinib daily. Among 267 patients with chronic-phase CML, 56% had a major cytogenetic response (51% of patients with resistance or intolerance to dasatinib or nilotinib and 70% of patients whose CML harbored the T315I mutation), 46% exhibited a complete cytogenetic response (40% and 66% in the two subgroups, respectively), and 34% had a major molecular response (27% and 56% in the two subgroups, respectively) [46]. However, following approval of ponatinib, a steady increase

in the number of serious vascular occlusion events identified through continued safety monitoring of the drug was noted. The drug was removed from the market. Per an agreement between the manufacturer and the FDA, the drug was reintroduced several months later with label changes to narrow the indication. The manufacturer was required to provide additional warnings about the risk of blood clots and severe narrowing of blood vessels, revised recommendations about the dosage and administration of ponatinib, and an updated patient Medication Guide. The manufacturer also was required to conduct post-market investigations to further characterize the drug's safety and dosing [47]. There was a phase III trial attempted which was the Evaluation of Ponatinib versus Imatinib in Chronic Myeloid Leukemia (EPIC) study, which was a randomized, open-label, phase III trial designed to assess the efficacy and safety of ponatinib, compared with imatinib, in newly diagnosed patients with chronic-phase chronic myeloid leukemia. However, the trial was terminated early, following concerns about vascular adverse events observed in patients given ponatinib in other trials [48]. There is currently an ongoing trial evaluating ponatinib at lower doses.

(iv) *Initial Dosage*: CML (refractory to all other TKIs or with T315I mutation): 45 mg once daily. Indicated in patients with the T315I polymorphisms and who have failed other TKIs.

(v) *Major Side Effects*: Major vascular events (see below), myelosuppression, pancreatitis, skin changes, abdominal pain, and headache.

(vi) *Reasons to Discontinue*: Vascular events, grade 3 or 4 myelosuppression, progression of disease, pancreatitis, and grade 3 elevations in transaminases (> five times the upper limit of normal).

(vii) *Black Box Warning*: Arterial occlusions have occurred in at least 30% of ponatinib-treated patients. Some patients experienced more than 1 type of event. Events observed included fatal myocardial infarction (MI), stroke, stenosis of large arterial vessels of the brain, severe peripheral vascular disease, and the need for urgent revascularization procedures. Patients with and without cardiovascular risk factors, including patients 50 years and younger, experienced these events. Monitor for evidence of arterial occlusion. Interrupt or stop ponatinib immediately for arterial occlusion. Heart failure, including fatalities, occurred in 9% of ponatinib-treated patients. Monitor cardiac function. Hepatotoxicity, liver failure, and death have occurred in ponatinib-treated patients. Monitor hepatic function. Interrupt ponatinib if hepatotoxicity is suspected. Venous occlusive events have occurred in 6% of ponatinib-treated patients. Monitor for evidence of venous thromboembolism. Consider dose modification or discontinuation of ponatinib in patients who develop serious venous thromboembolism.

(f) *Omacetaxine*

(i) *Brand Name*: Synribo

(ii) *Classification/MOA*: Protein synthesis inhibitor

(iii) *Relevant Studies*: In a phase II study of subcutaneous omacetaxine mepesuccinate after TKI failure in patients with chronic-phase CML with T315I mutation, complete hematologic response was achieved in 77% (95% lower confidence limit, 65%). 23% (95% lower confidence limit, 13%) achieved major cytogenetic response, including complete cytogenetic response in 16%. Median progression-free survival was 7.7 months [49]. There was a second phase II study by the same group looking at subcutaneous omacetaxine mepesuccinate for chronic-phase chronic myeloid leukemia patients resistant to or intolerant of tyrosine kinase inhibitors. Eighteen percent of chronic phase CML patients achieved a major cytogenetic response (MCyR) with a median duration of 12.5 months (95% confidence interval [CI], 3.5 months to not reached [NR]), and the median overall survival (OS) was 40.3 months (95% CI, 23.8 months to NR). Among patients with accelerated phase CML, 14% achieved or maintained a major hematologic response for a median of 4.7 months (95% CI, 3.6 months to NR); MCyR was not achieved, and the median OS was 14.3 months (95% CI, 6.7–18.7 months) [50].

(iv) *Major Side Effects*: Myelosuppression, diarrhea, nausea, fatigue, fever, and headache.

(v) *Reasons to Discontinue*: Progression of disease, grade 3 or 4 myelosuppression, and intolerance of non-hematologic side effects.

Summary

Chronic myeloid leukemia (CML) is a malignant disease of hematopoietic progenitor cells. It arises from a t(9;22) which is a reciprocal chromosomal translocation between the ABL proto-oncogene and the BCR gene on chromosome 22. The resulting product (BCR-ABL1) is a constitutively active tyrosine kinase leading to uncontrolled proliferation of myeloid, monocytic, erythroid, megakaryocytic, B-lymphoid, and occasionally T-lymphoid cell lineages. CML can be classified as being in chronic, accelerated, or blastic phase which impacts both treatment and prognosis. For patients in chronic

phase, once the diagnosis is confirmed, therapy with a tyrosine kinase inhibitor (TKI) should be started. Patients should then be monitored serially with a hematologist following ELN criteria. If patients progress or lose their response, adherence to therapy should be investigated and a BCR-ABL1 mutational panel sent to guide the next step in therapy. For those that remain in remission, there is emerging data that TKI therapy can be safely discontinued in a subset of patients who have achieved deep remission and maintained it for several years. For patients presenting in accelerated phase and blast crisis, treatment remains challenging even in the age of TKIs. Generally, patients are first given induction therapy followed by allogeneic hematopoietic cell transplantation (HCT), although, in the era of increasing TKI options, there has not been a randomized trial looking at HCT versus continuing to the next TKI if response is lost.

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Myeloproliferative Neoplasms

26

Emma J. Gudgin and Anna L. Godfrey

Introduction

The myeloproliferative neoplasms (MPNs) are clonal diseases of hematopoietic stem cells causing abnormal proliferation of fully differentiated myeloid blood cells. This leads to an increase in primarily red blood cells in polycythaemia vera (PV) and platelets in essential thrombocythaemia (ET), although there can be proliferation of all myeloid cell types in any of the MPNs. Myelofibrosis may arise de novo (primary myelofibrosis, PMF) or progress from PV or ET. Less common MPNs are caused by clonal proliferations of eosinophils or neutrophils (Table 26.1). Chronic myeloid leukaemia (CML) caused by the BCR-ABL1 translocation is considered in Chap. 25. Mastocytosis, caused by a clonal proliferation of mast cells, is classified as a separate entity but is related to the MPNs.

A major step in understanding the molecular basis of non-CML MPNs came in 2005 with the discovery that more than 95% of patients with PV and approximately 50% of those with ET and PMF carried a single point mutation (*V617F*) in the gene coding for JAK2. JAK2 is a cytoplasmic tyrosine kinase that acts downstream of multiple cytokine receptors, including those for erythropoietin and thrombopoietin (which drive production of red cells and platelets, respectively). The *V617F* mutation results in constitutive activation of JAK2 in the absence of normal ligands, leading to excessive activation of downstream signaling pathways. Many patients with PV who lack the *V617F* mutation have an alternative mutation in exon 12 of the gene, again leading to constitutive activation of the protein. Patients with ET and PMF who lack *JAK2 V617F* may have a mutation in either the *CALR* gene, which encodes an endoplasmic reticulum protein chaperone protein, or in *MPL*, the gene for the thrombopoietin receptor; both of these mutation types seem to act through activation of pathways downstream of the thrombopoietin receptor. Identification of these and other mutations and translocations have now clarified the molecular pathogenesis of the majority of MPNs (Table 26.2).

Table 26.1 2016 World Health Organization classification of MPNs (excluding CML)

Polycythaemia vera (PV)
Essential thrombocythaemia (ET)
Primary myelofibrosis (PMF)
Chronic neutrophilic leukaemia (CNL)
Chronic eosinophilic leukaemia, not otherwise specified (CEL, NOS)
Myeloproliferative neoplasm, unclassifiable (MPN, unclassifiable)

MPNs may be diagnosed following incidental findings on a full blood count, but other presenting features and complications include arterial and venous thrombosis. The possibility of an underlying MPN should be considered in patients presenting with an unprovoked thrombosis, particularly of an unusual site such the portal or hepatic vein. Bleeding complications, usually in the context of a high platelet count, can also occur as a result of platelet dysfunction. Constitutional symptoms including weight loss, fatigue, pruritus, fever, night sweats and bone pain may develop. Gout is more prevalent in all MPNs due to increased cellular proliferation. Splenomegaly can also occur in all MPNs but is most often clinically evident in myelofibrosis.

Investigations of a patient with a suspected MPN are focused on distinguishing the presenting features (e.g. erythrocytosis or thrombocytosis) from non-clonal, reactive causes of an abnormal blood count and then in establishing the correct sub-classification of disease and prognostic

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Table 26.2 Molecular abnormalities in the MPNs

WHO category	Incidence	Pathogenetic abnormalities
Polycythaemia vera	2–2.8/100,000	JAK2 V617F: 95–97%
		JAK2 exon 12: 3%
Essential thrombocythaemia	1.5–2.4/100,000	JAK2 V617F: 50–60%
		CALR: ~20–25%
		MPL: 4–5%
		Unknown in ~15%
Primary myelofibrosis	0.5–1.3/100,000	JAK2 V617F: ~50%
		CALR: ~30%
		MPL: 5–10%
Myeloid and lymphoid neoplasms associated with eosinophilia and abnormalities of PDGFRA, PDGFRB, FGFR1 or PCM1-JAK2		FIP1L1-PDGFRA, PDGFRB, FGFR1 or PCM1-JAK2
Chronic neutrophilic leukaemia	Very rare	CSF3R: ~90%
Chronic eosinophilic leukaemia, NOS	Rare	None specific
Mastocytosis	Unclear	KIT: >95%

factors. Aims of treatment, in general, are to minimize risks of thrombosis and bleeding and to address constitutional symptoms. A minority (5–15%) of patients with ET and PV develop transformation to myelofibrosis, and all of these diseases can transform to acute myeloid leukaemia, but current treatment options are unable to prevent disease progression.

Erythrocytosis and Polycythaemia Vera

Presentation and Investigation

The haematocrit (HCT) defines the cellular compartment of the blood, which mostly consists of red blood cells. A raised HCT is considered significant if persistent, for example, >52% (0.52) in men or >48% (0.48) in women. This may be due to an increase in red cells, i.e. true erythrocytosis, or may be "apparent", due to a reduction in plasma volume. A true erythrocytosis may result from a MPN (i.e. PV) or from secondary causes or represent idiopathic erythrocytosis. There are many secondary causes of erythrocytosis (Table 26.3), the most common being hypoxic lung disease and renal diseases.

Investigation of an erythrocytosis (Fig. 26.1) begins with a clinical history and examination to identify causes of apparent or secondary erythrocytosis, including any family history of erythrocytosis. First-line investigations include mutation screening for *JAK2 V617F* (plus exon 12 mutations if *V617F* is undetectable), serum ferritin and renal function. In practice, a *JAK2* mutation with a significantly raised haematocrit (>49% in men, >48% in women) or Hb >165 g/L (>16.5 g/dL) in men and >160 g/L (>16.0 g/dL) in women is often considered sufficient to diagnose PV, although a bone marrow aspirate and trephine biopsy are frequently performed at diagnosis. *JAK2* mutation-negative PV is very rare and requires supportive bone marrow histology in addition to a low serum erythropoietin level. True erythrocytosis with no primary or secondary cause defines idiopathic erythrocytosis.

Table 26.3 Causes of secondary erythrocytosis

Acquired
Chronic hypoxia
Lung disease
Obstructive sleep apnoea
Congenital cyanotic heart disease
Heavy smoking
Renal disease causing local hypoxia (e.g. renal cysts, renal artery stenosis)
Pathological erythropoietin production
Renal tumours
Other tumours (e.g. cerebellar haemangioblastoma, hepatoma)
Drug associated
Androgenic steroids (e.g. testosterone preparations)
Erythropoietin use
Familial/inherited
High oxygen affinity haemoglobin
2,3-Bisphosphoglycerate mutase deficiency
Mutations in the oxygen sensing pathway, e.g. *VHL* gene
Erythropoietin receptor-mediated

Management

Management of secondary erythrocytosis is largely focused on addressing the underlying cause (e.g. oxygen therapy in chronic obstructive pulmonary disease), although occasional patients require venesection to reduce their risk of thrombosis.

The management of PV centres on prevention of cardiovascular events. All patients should receive aspirin thromboembolic prophylaxis, unless there is a contraindication to this intervention, since there is evidence that aspirin reduces cardiovascular events in patients with PV. Other cardiovascular risk factors including diabetes mellitus, essential hypertension and hypercholesterolaemia should be managed aggressively, and patients should be given lifestyle advice including smoking cessation. Patients who have suffered a venous thrombosis should be managed with anticoagulation, which is often indefinite, particularly in cases of unprovoked or life-threatening thrombosis.

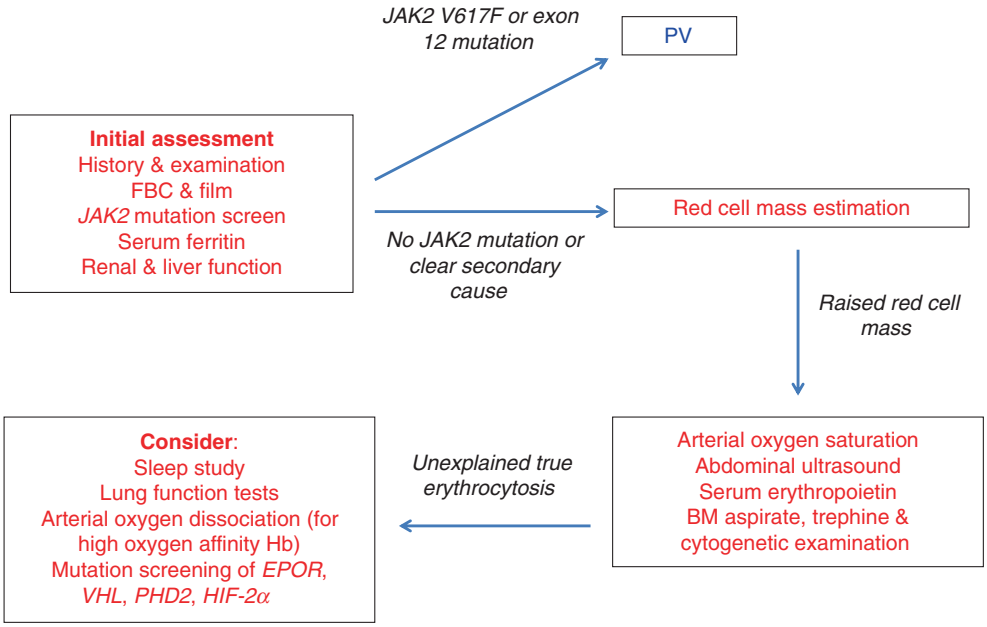

Fig. 26.1 Investigation of erythrocytosis

Controlling the haematocrit to a target of <45% reduces the risk of vascular events in patients with PV. In the first instance, including during diagnostic workup, it is appropriate to use venesection procedures to reduce the haematocrit. Venesection is typically performed once or twice weekly initially, but more frequent procedures may be indicated in patients who have a particularly high haematocrit at diagnosis or those at high vascular risk (e.g. a recent thrombosis). In patients considered at lower risk of thrombosis (age <60 years and no history of thrombotic events), it is often appropriate to continue to manage the haematocrit with venesection alone; the frequency of procedures usually decreases over time, as patients become deficient in iron (e.g. every 1–3 months).

In some patients, repeated venesection is technically difficult or cannot be tolerated, or is inadequate to control the haematocrit, or leads to a marked thrombocytosis. In these patients and in those at higher risk of thrombosis (age >60 years and/or previous thrombosis), it is appropriate to use cytoreductive drugs to control the haematocrit. These treatments can also be helpful for patients who have marked constitutional symptoms (e.g. sweats), symptomatic splenomegaly or progressive leucocytosis. The most commonly used agent is hydroxycarbamide (hydroxyurea), a ribonucleotide reductase inhibitor that is usually well tolerated. Its side effects include cytopenias, mouth ulcers and occasionally leg ulcers. Interferon-alfa is an alternative first-line therapy, which is the only cytoreductive agent known to be safe in pregnancy in PV, and is often used in younger patients. Its use is limited, particularly in older patients, by a higher rate of side effects including flulike symptoms, bone pain, cytopenias, depression and hypothyroidism. It usually requires daily or alternate daily self-injection, but more recently pegylated interferon-alfa preparations have been used, and these have the advantages of weekly administration and better tolerability.

Most recently JAK2 inhibitors have also been shown to have benefit in controlling blood counts and splenomegaly in patients with PV who are resistant or intolerant of hydroxycarbamide.

Thrombocytosis and Essential Thrombocythaemia

Presentation and Investigation

A raised platelet count (thrombocytosis) is most commonly a reaction to infection, inflammation, iron deficiency or recent surgery but may also be due to a MPN. Any MPN may be associated with a thrombocytosis, but it is the most prominent feature of essential thrombocythaemia (ET). Presentation of ET is often incidental, but 30–50% of patients with ET have microvascular occlusive events (e.g. burning pain in the extremities – erythromelalgia – or digital ischaemia), major vascular occlusive events or haemorrhage at presentation. A persistent platelet count above 450×10^9/l should be investigated as shown in Fig. 26.2.

Initial assessment aims to exclude reactive causes of thrombocytosis. The molecular pathogenesis of approximately 85% of cases of ET is understood, with a *JAK2 V617F* mutation found in about 50% of cases of ET and mutations in the *CALR* and *MPL* genes in a smaller proportion (Table 26.1). Occasionally patients with chronic myeloid leukaemia will present with an isolated thrombocytosis, so a *BCR-ABL* fusion should be excluded in patients without a

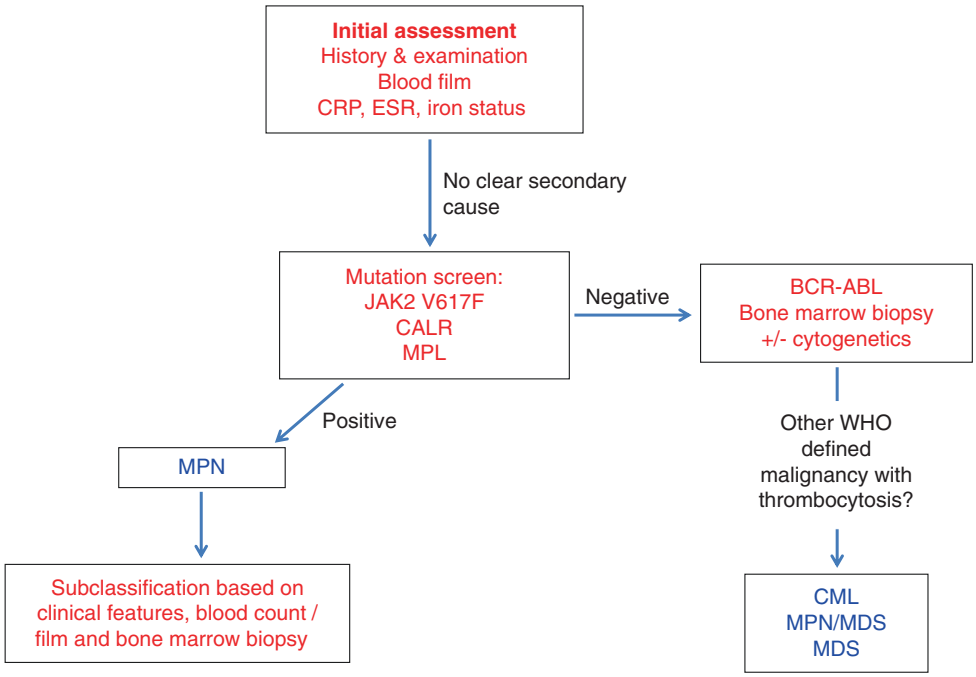

Fig. 26.2 Investigation of thrombocytosis

known mutation. A bone marrow trephine is required to make a diagnosis of ET, both to confirm that features of a MPN are present where no molecular abnormality is found, but also to exclude primary myelofibrosis. Trephine histology often reveals features such as atypical, abnormally large megakaryocytes that are suggestive of ET.

Management

Reactive thrombocytosis does not generally require specific management and will resolve when the underlying cause is addressed. It is not generally associated with an increased risk of thrombosis, although patients in the post-surgical setting should receive thromboprophylaxis appropriate for their risk of venous thromboembolism. In patients with MPNs especially, splenectomy can be associated with a marked thrombocytosis, and this can precipitate thrombosis, so cytoreduction should be used to control the platelet count.

Treatment of ET utilizes the same principles as that of PV. Management of other cardiovascular risk factors is essential. Most patients are given aspirin, although caution is required in patients with the highest platelet counts ($>1000\times10^9$/l) because of the increased risk of bleeding. ET is a heterogeneous disease with respect to the risk of cardiovascular events, and patients are stratified according to this risk. Currently patients aged >60 years and those with a prior history of thrombosis, or of haemorrhage due to ET, are managed with cytoreductive drugs with the aim of keeping the platelet count in the normal range. Patients with a platelet count $>1{,}500\times10^9$/l are also given cytoreduction, principally because of the high risk of haemorrhage associated with extreme thrombocytosis. Patients with ET and a *JAK2* mutation have a higher risk of thrombosis than those with a *CALR* mutation, but mutation status is not yet a factor used routinely to determine whether cytoreduction is indicated.

In patients who require treatment to reduce the platelet count, hydroxycarbamide is the most common first-line agent, as in PV. Anagrelide is an agent that tends to act specifically on the platelet count and can therefore be useful in ET. It is associated with a slightly higher risk of arterial thrombosis and of transformation to myelofibrosis than hydroxycarbamide so is generally used second line. Interferon-alfa is used for some younger patients, particularly in those planning pregnancy.

Primary Myelofibrosis

Presentation

Primary myelofibrosis (PMF) is a disorder of increased fibrosis within the bone marrow, accompanied by a number of characteristic clinical features. The patient may present with symptoms of cytopenias (most often anaemia), with symptoms of splenomegaly such as abdominal discomfort or early satiety or with constitutional symptoms such as fatigue, weight loss and night sweats. Clinical examination often reveals splenomegaly which can be massive and is sometimes accompanied by hepatomegaly; these are a consequence of extramedullary haematopoiesis, in which blood cells are produced outside the bone marrow.

Whilst anaemia is common, platelet and white cell counts may be high, normal or low at presentation. The peripheral blood film typically shows a "leucoerythroblastic" picture with immature granulocytic and erythroid cells that are not usually found in the blood, usually accompanied by teardrop-shaped red blood cells. A few cases present with an incidental finding of an abnormal blood count or film, although patients with myelofibrosis are more likely to be symptomatic than those with PV or ET. Patients with PV or ET who progress to myelofibrosis (post-PV or post-ET MF) may develop the same clinical features.

Diagnosis

The diagnosis of PMF is made when clinical suspicion prompts examination of a peripheral blood film and a bone marrow biopsy. It should be noted that a leucoerythroblastic blood film is not specific to the diagnosis of PMF but can be found in other diseases characterized by an infiltration of the bone marrow (e.g. metastatic malignancy), other primary bone marrow disorders (e.g. chronic myeloid leukaemia) or conditions characterized by bone marrow stress (e.g. severe sepsis or haemolysis). Aspiration of liquid bone marrow is often difficult in the presence of significant fibrosis (known as a "dry tap"), and examination of the solid bone marrow trephine specimen is required to recognize the characteristic histological features. The finding of increased bone marrow fibrosis does not only occur in PMF and must be distinguished from secondary causes of bone marrow fibrosis, including metastatic malignancy and certain autoimmune disorders.

Together with the characteristic histologic findings and molecular abnormalities, additional clinical features must also be present and international diagnostic criteria for PMF have been developed to incorporate these (Table 26.4). The finding of a *JAK2*, *CALR* or *MPL* mutation can strongly support a diagnosis of PMF, but it must be remembered that 10–15% of patients with PMF do not have one of these mutations. Recurrent mutations in other genes have also been reported in patients with PMF, such as in *ASXL1*, *IDH1/2* and *EZH2*. The finding of one of these latter mutations again supports the diagnosis of a clonal haematologic disorder (e.g. rather than reactive marrow fibrosis), although they are not specific to MPNs and are found in other myeloid malignancies such as MDS and AML.

Table 26.4 World Health Organization criteria for the diagnosis of primary myelofibrosis (2016). Diagnosis of overt primary myelofibrosis (PMF) requires all three major criteria and at least one minor criterion

Major criteria	Minor criteria
(1) Increased megakaryocytes with typical abnormal features; increased bone marrow fibrosis (reticulin and/or collagen)	(1) Unexplained anaemia
(2) Not meeting criteria for another myeloid neoplasm (e.g. CML, ET, PV, MDS)	(2) Leucocytosis $\geq 11 \times 10^9$/L
(3) *JAK2*, *CALR* or *MPL* mutation; in the absence of these mutations, another clonal marker (e.g. *ASXL1* mutation) or absence of reactive myelofibrosis	(3) Splenomegaly (palpable)
	(4) Increased serum lactate dehydrogenase
	(5) Leucoerythroblastic blood film

Prognosis

The median survival of PMF is 5–6 years but varies widely between patients. Certain prognostic factors are known to be associated with a poorer prognosis, including anaemia and thrombocytopenia (Table 26.5). More recently certain molecular abnormalities, such *ASXL1* mutations, have also been shown to confer a worse prognosis.

Management

PMF, post-PV MF and post-ET MF are heterogeneous diseases, and management of an individual patient depends on their specific clinical presentation.

Allogeneic Hematopoietic Cell Transplantation

The only potentially curative treatment is allogeneic hematopoietic cell transplantation. This procedure carries a significant risk of mortality and morbidity (see Chap. 37) and is therefore only suitable for the minority of patients who present at a younger age, without significant comorbidities, with an appropriate hematopoietic cell donor and with disease that has sufficient poor prognostic factors to warrant the risk of transplantation. In all other patients, treatment is supportive and tailored towards their specific symptoms.

Splenomegaly

In patients with symptomatic splenomegaly, a novel class of drugs that inhibit JAK2 has been shown to reduce spleen size and improve symptoms. These inhibitors, the first of which was ruxolitinib, are not specific for V617F-mutated JAK2 (and some also inhibit other related kinases such as JAK1). JAK2 inhibitors do not reverse the underlying disease process

Table 26.5 Poor prognostic features in myelofibrosis

Age >65 years
Constitutional symptoms (fevers, night sweats, weight loss)
Haemoglobin <100 g/l (especially if transfusion-dependent)
White cell count $>25 \times 10^9$/l
Blast cells ≥1% in peripheral blood
Platelet count $<100 \times 10^9$/l
Unfavourable bone marrow chromosomal abnormalities

nor reduce the risk of the myelofibrosis progressing to acute leukaemia. They generally do not improve anaemia or thrombocytopenia and can, in fact, worsen these, so must be used with caution in patients with significant cytopenias. However, they can provide important symptomatic relief of splenomegaly and constitutional symptoms and have been associated with an improvement in survival.

Prior to the development of JAK2 inhibitors, other drugs were used for splenomegaly, most commonly hydroxycarbamide, which can also control high white cell and/or platelet counts that are found in some patients with myelofibrosis. Splenectomyhas been used for patients with drug-refractory symptomatic splenomegaly but carries a significant risk of mortality and morbidity including postoperative thrombosis. Less commonly, splenic radiotherapy has also been used and can be effective, but its benefits are generally temporary. These options are used much less frequently in the era of JAK2 inhibitors.

Constitutional Symptoms

Symptoms such as fatigue, weight loss, sweats, pruritus and bone pain are common in PMF. JAK2 inhibitors are particularly effective in controlling these symptoms and have been associated with improvements in quality of life scores.

Cytopenias

In patients with significant anaemia, either recombinant erythropoietin or the synthetic androgen danazol can sometimes improve haemoglobin levels. However, the majority of patients do not respond, and most with symptomatic anaemia require support with regular transfusions. Mild to moderate thrombocytopenia is not usually associated with symptoms. In patients with severe thrombocytopenia (platelet count $<10–20\times10^9$/L), tranexamic acid can be used for mucosal bleeding, although the patient's individual thrombotic risk should be considered when using this anti-fibrinolytic agent. Occasionally platelet transfusions are required for clinically significant bleeding episodes.

Blast Phase

Blast phase refers to the progression of myelofibrosis (primary, post-PV or post-ET) such that diagnostic criteria for acute myeloid leukaemia are met (generally >20% blasts in peripheral blood or bone marrow). This phase of disease has a poor prognosis, usually less than 12 months and frequently shorter. In a small minority of patients, intensive chemotherapy can be attempted to control the disease, prior to allogeneic hematopoietic cell transplantation which can be curative. However, in the majority of patients, care is supportive, focusing on management of symptoms including transfusion support for cytopenias.

Eosinophilia

Presentation and Differential Diagnosis

Eosinophilia is a raised eosinophil count, $>0.5\times10^9$/l. Eosinophils are usually found in the blood and bone marrow, lymphoid tissues and the GI tract. Invasion of other tissues can occur with prolonged eosinophilia, potentially leading to damage of the heart, lungs, nervous system or rashes. Venous thromboembolic disease can also be a manifestation of eosinophilic tissue damage.

Eosinophilia is a relatively common finding, most commonly secondary to one of a large number of conditions (Table 26.6). These include malignant conditions such as Hodgkin lymphoma, in which 15% of patients have a secondary, non-clonal eosinophilia. Eosinophilia may also be a primary, clonal disorder; here the eosinophil count is often higher than reactive eosinophilias, although this is not always the case.

1. Myeloid/Lymphoid Neoplasms with Eosinophilia and Rearrangements of PDGFRA, PDGFRB, FGFR1 or PCM1-JAK2

Clonal eosinophilia is often driven by tyrosine kinase gene fusions. These typically involve rearrangements in the genes coding for platelet-derived growth factor receptor alpha (*PDGFRA*), platelet-derived growth factor receptor beta (*PDGFRB*) or fibroblast growth factor receptor 1 (*FGFR1*). They may be associated with either myeloid or lymphoid neoplasms, such as acute lymphoblastic leukaemia (ALL). Less commonly translocations involve genes for other tyrosine kinases such as JAK2 (note that these translocations are different from the V617F and exon 12 mutations seen in PV) or FLT3.

2. Chronic Eosinophilic Leukaemia and Other Clonal Causes of Eosinophilia

Clonal eosinophilia may also be present in other myeloid neoplasms. CML (Chap. 25) is often associated with an eosinophilia, alongside left-shifted myeloid precursors (i.e. immature granulocytes such as myelocytes) and basophilia in chronic phase. Acute myeloid leukaemia (AML) with chromosome translocations t(8;21) or inversion 16 are often associated with an eosinophilia, which is part of the leukaemic clone. Systemic mastocytosis is often accompanied by an eosinophilia, which may be clonal, reactive or a mixture

Table 26.6 Secondary (reactive) causes of eosinophilia

Cause/system		Investigations
Allergic	Asthma Eczema Allergic rhinitis (hay fever)	Serum immunoglobulin E (IgE) Allergen-specific IgE tests Skin prick testing
Drugs	For example, antibiotics, anticonvulsants	Thorough drug history
Infectious diseases	Strongyloides Schistosomiasis Filariasis Toxocariasis	Fresh stool microscopy for ova, cysts and parasites Serological tests for suspected parasitic infections Consider HIV and HTLV1 testing if opportunistic infection
Gastrointestinal	Primary gastrointestinal eosinophilic disorders including eosinophilic oesophagitis Chronic pancreatitis Inflammatory bowel disease Coeliac disease	Upper gastrointestinal endoscopy, small bowel endoscopy and/or colonoscopy/sigmoidoscopy Serum amylase Serology for coeliac disease-related autoantibodies
Vasculitides	Polyarteritis nodosa Eosinophilic granulomatosis with polyangiitis (Churg-Strauss syndrome)	ANCA, tissue biopsy Serology for hepatitis B,C HIV and parvovirus
Rheumatological	Systemic lupus erythematosus Rheumatoid arthritis	ANA Anti-ds-DNA
Respiratory	Acute and chronic eosinophilic pneumonias (including Löffler syndrome) Allergic bronchopulmonary aspergillosis Sarcoidosis	Imaging Bronchoscopy and lavage Aspergillus-specific IgG Serum IgE Aspergillus skin prick test Serum ACE Tissue biopsy

of both. Chronic eosinophilic leukaemia (CEL) is a clonal eosinophilia without a tyrosine kinase fusion, but with either an increase in bone marrow blasts or another clonal marker such as trisomy 8.

Idiopathic hypereosinophilia is a diagnosis that requires exclusion of all primary and secondary causes of eosinophilia, with an eosinophil count at least 1.5×10^9/l and no evidence of clonality or a reactive cause. If eosinophil-mediated tissue damage is also present, for example, affecting the heart, lungs, skin, nervous system or gastrointestinal tract, this is called idiopathic hypereosinophilic syndrome.

Investigation

The investigation of eosinophilia is outlined in Fig. 26.3. Beyond the initial investigations, if the eosinophil count is $<1.5 \times 10^9$/l and there is no evidence of systemic symptoms or organ damage, no further testing may be indicated. With higher eosinophil counts, or if there is a clinical suspicion of eosinophil-related symptoms or toxicity, further investigations for primary and secondary causes of eosinophilia should be pursued.

Management

The management of eosinophilia depends on the cause and the presence of symptoms or organ damage. Emergency treatment is with corticosteroids and may be required in situations where there is eosinophil-mediated tissue damage, whilst the cause is being investigated. Secondary eosinophilia is generally treated by managing the underlying condition.

In cases of clonal eosinophilia due to tyrosine kinase rearrangements in which there is a specific target, an appropriate tyrosine kinase inhibitor can be used. This highlights the importance of excluding these abnormalities prior to reaching a diagnosis of the cause of the eosinophilia. For example, the tyrosine kinase inhibitor imatinib is used where a PDGFRA or PDGFRB translocation is present, whereas the JAK2 inhibitor ruxolitinib is used in cases with JAK2 translocations. Clonal eosinophilia associated with other haematological neoplasms is treated according to the underlying cause; although if tissue damage due to the eosinophilia is present, the addition of corticosteroids can be considered.

Patients with clonal eosinophilia with no molecular target or idiopathic hypereosinophilia are treated initially with corticosteroids. There is no definite consensus on the eosinophil level requiring initiation of therapy in an asymptomatic patient, as the eosinophil count correlates poorly with the development of organ damage. In steroid-refractory patients, imatinib may be trialled for a short number of weeks as it has been shown to have efficacy in some patients, particularly those with myeloproliferative features. Other immunomodulatory agents such as interferon-alfa may also be used.

Some clonal eosinophilias, such as those with FGFR1 translocations, have an aggressive disease course. In these cases more intensive combination chemotherapy regimens (similar to those used for remission induction in acute leukaemia) may be considered, followed by allogeneic haematopoietic cell transplantation.

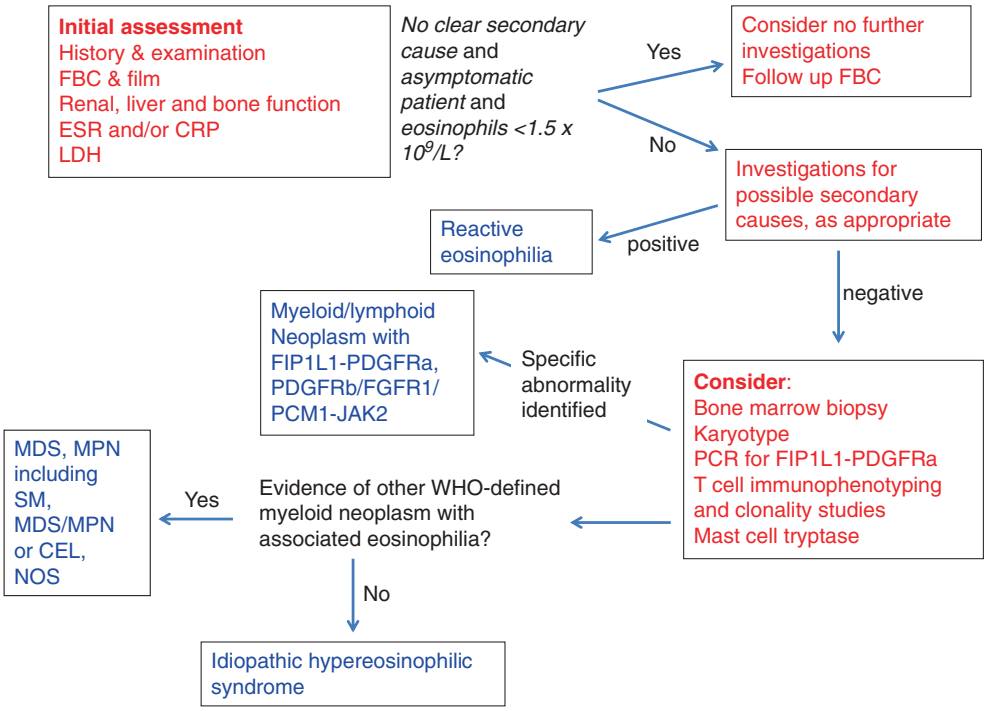

Fig. 26.3 Investigation of eosinophilia

Mastocytosis

Presentation

Mastocytosis refers to a collection of rare disorders, all characterized by accumulation of abnormal mast cells. The group is divided into cutaneous mastocytosis (CM), in which disease is predominantly limited to the skin, and systemic mastocytosis (SM), in which bone marrow and/or other extracutaneous tissues are infiltrated. Whilst CM is commoner in children, in many of whom the skin lesions will regress around puberty, SM is most often a disease of adulthood that does not resolve spontaneously.

Patients with SM present with clinical features that can broadly be divided into two groups: those that result from the release of vasoactive mediators from mast cells and those resulting from damage to the organs infiltrated by mast cells. Mediator release can cause skin symptoms (pruritus, urticaria, flushing), gastrointestinal disturbances (nausea, vomiting, diarrhoea, abdominal cramps, dyspepsia), cardiovascular effects (dizziness, palpitations, collapse) and neurological symptoms (headache, memory impairment). Some patients develop recurrent anaphylaxis, which may be triggered by specific foods, drugs or insect stings or other constitutional symptoms (weakness, fatigue, sweats, myalgias). Effects on bone include osteopenia, osteoporosis or sometimes large lytic lesions that can be associated with pain or fractures. Other organ infiltration can cause splenomegaly, hepatomegaly and ascites, lymphadenopathy, cytopenias and malabsorption.

Diagnosis

Diagnosis of SM depends on the demonstration of mast cell infiltrates on tissue biopsy, together with additional findings that indicate the neoplastic nature of the cells (Table 26.7). Patients may be investigated following presentation with a typical skin rash, or in patients with typical mediator-release symptoms, the finding of a persistently raised serum tryptase would prompt further investigations. The bone marrow is frequently affected, and bone marrow biopsy is performed in all patients suspected to have SM. The vast majority of adult patients have a mutation in the *KIT* gene (most often *D816V*, although other mutations have been identified), leading to activation of the KIT tyrosine kinase which acts as the receptor for stem cell factor.

SM is further subclassified according to the burden of abnormal mast cells in organs and the severity of clinical features (Table 26.7). A subgroup of patients with SM presents with features of another haematologic neoplasm (SM-AHN), most often a myeloid neoplasm such as an MDS/MPN overlap disorder, another MPN or MDS. These patients often have other molecular abnormalities such as *JAK2 V617F*, *TET2*, *ASXL1* or *SRSF2* mutations, which are typical of the other haematologic disorder.

Management

Treatments for cutaneous mastocytosis include avoidance of triggering factors, topical therapies (e.g. topical corticoste-

Table 26.7 Summary of diagnostic criteria in systemic mastocytosis

Diagnosis	Criteria required	Details
Systemic mastocytosis (SM): all patients	Major + 1 minor criteria or at least 3 minor criteria	Major: multifocal, dense aggregates of ≥15 mast cells in bone marrow or other extracutaneous organ
		Minor (1): atypical (spindle-shaped) mast cell appearance
		Minor (2): activating *KIT D816* mutation
		Minor (3): CD2 and/or CD25-positive mast cells
		Minor (4): persistently elevated serum tryptase (>20 ng/mL) [criterion 4 is not valid in SM-AHM]
Subcategories of SM		
Indolent SM	Does not meet criteria for subcategories below	
Smouldering SM	At least 2 "B" findings	B findings
		(1) Bone marrow biopsy shows >30% mast cell infiltration and/or serum tryptase >200 ng/mL
		(2) Dysplasia or myeloproliferation in bone marrow but not meeting diagnostic criteria for another neoplasm
		(3) Hepatomegaly without impaired liver function/palpable splenomegaly without hypersplenism/lymphadenopathy
Aggressive SM	At least 1 "C" finding	C findings
		(1) Cytopenias (Hb <100 g/l / neutrophils $<1.0\times10^9$/l / platelets $<100\times10^9$/l)
		(2) Palpable hepatomegaly with impaired liver function/ascites/portal hypertension
		(3) Large osteolytic lesions in the bone and/or fractures
		(4) Palpable splenomegaly with hypersplenism
		(5) Malabsorption with weight loss
SM-AHN	Meets criteria for SM + another haematological neoplasm	
Mast cell leukaemia	≥20% mast cells in bone marrow smears	

AHN associated haematological neoplasm

roid) and systemic therapies (e.g. antihistamines, sodium cromoglycate). Management of SM can be divided into treatment of mediator-release symptoms and therapies to reduce the mast cell burden. Patients with SM-AHN should be treated for the associated haematologic neoplasm, alongside treatment for the SM as appropriate.

Mediator-Release Symptoms

Mild symptoms can be managed with a combination of histamine receptor (H1 and H2) antagonists, but additional drugs may be required for more severe symptoms. Corticosteroids, mast cell stabilizers and leukotriene antagonists can be helpful. Patients with anaphylaxis are trained to carry and use adrenaline self-injectors. Gastrointestinal symptoms may respond to proton pump inhibitors. Patients with osteoporosis are treated with bisphosphonates but do not always improve, and other drugs such as interferon-alfa may be required. Patients are also educated to avoid triggers of mast cell degranulation including certain drugs.

Reducing Mast Cell Burden

In patients with aggressive SM, and in some with smouldering SM and severe symptoms, additional therapy is indicated to reduce the neoplastic mast cells. Options include interferon-alfa, the tolerability of which is limited by side effects (see above). Cladribine is an alternative cytoreductive agent with potential toxicities including bone marrow suppression, lymphopenia and increased risk of opportunistic infections. Patients with the most aggressive disease (e.g. mast cell leukaemia) require combination chemotherapy. Some novel agents that inhibit KIT are also available (e.g. midostaurin), and these may benefit patients with advanced disease.

Conclusions

The non-CML MPNs share certain haematologic features but are clinically heterogeneous in their clinical presentation, prognosis and management. Most importantly the molecular basis of most of these of disorders is now established and can be confirmed in patient samples using relatively simple molecular and cytogenetic techniques. These assays have been integrated into routine clinical practice and have greatly simplified diagnostic pathways for patients presenting with possible features of a MPN. In a few cases, these molecular lesions can now be targeted by specific pharmaceutical agents. However therapeutic options for the commonest disorders such as PV, ET and PMF remain suboptimal, with a lack of drugs that can reverse either the underlying disease

process or the risk of progression to acute myeloid leukaemia. Future research will aim to address these therapeutic limitations and to establish further how additional molecular abnormalities contribute to the variable clinical phenotypes that are seen within, and between, disease categories of the MPNs.

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Myelodysplastic Syndromes (MDS)

27

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Introduction and Pathogenesis

Myelodysplastic syndromes (MDS) are a group of clonal hematopoietic bone marrow stem cell neoplasms that are characterized by ineffective formation of peripheral blood cells resulting in one or more cytopenias (anemia, neutropenia, and/or thrombocytopenia). MDS causes morphologic abnormalities in the bone marrow cell lines, described as "dysplastic changes." As a consequence of cytopenias, patients often require red blood cell and/or platelet transfusion support and are at a higher risk of developing infections. In addition, these neoplastic stem cell clones have the ability to undergo progressive molecular evolution, leading to acute myeloid leukemia (AML) in 25–33% of patients. The pathogenesis of MDS is thought to be due to acquiring oncogenic driver mutations over time. Mutations associated with MDS occur de novo or, in approximately 10% of patients, secondary to prior exposure to chemotherapy, radiation therapy, environmental toxins such as benzene (as might occur in the rubber industry), or smoking [20, 26, 28, 33].

Epidemiology

The incidence of MDS increases with age; it is uncommonly diagnosed before the age of 50 years [17]. The median age at diagnosis is 71 years [28, 29]. The age-adjusted yearly incidence rate of MDS is 4.9 per 100,000 per surveillance, epidemiology, and end results (SEER) data, with significantly higher numbers in males and in white, non-Hispanic patient populations [15]. This information is widely considered to be an underestimate, with reanalyses of the SEER data based on insurance claims demonstrating an incidence rate that may be as high as 75 per 100,000 in patients ≥65 years of age [6]. Certain familial disorders predispose to MDS, such as Diamond-Blackfan anemia, severe congenital neutropenia, Shwachman-Diamond syndrome, dyskeratosis congenita, Fanconi anemia, and Bloom syndrome, along with myeloid neoplasms with specific molecular abnormalities, such as those involving germline mutations in the gene *DDX41* (dead-box helicase 41), involved in RNA strand separation [23, 24].

Clinical Presentation

Patients have a variety of clinical presentations ranging from being completely asymptomatic and having incidental laboratory abnormalities on routine blood work to presenting with significant signs or symptoms as a result of low blood counts. Patients with anemia often complain of fatigue but may also have shortness of breath, light-headedness, decreased exercise tolerance, or chest pain. Patients with thrombocytopenia may present with easy bruising and/or other bleeding complications such as petechiae, epistaxis, or even gastrointestinal bleeding or stroke. Infection risk in the setting of neutropenia is a well-recognized source of morbidity and is the most common cause of mortality in MDS [7]. The risk of infections increases with absolute neutropenia and due to qualitative neutrophil defects. Neutrophil dysfunction is secondary to abnormal phagocytosis, decreased bactericidal and fungicidal activities, and defective chemotaxis [35]. Splenomegaly may be found in patients with MDS/myeloproliferative neoplasms (MPN). These overlap syndromes include clinical, lab, and morphologic features of both MDS and MPN such as chronic myelomonocytic leukemia (CMML) or atypical chronic myeloid leukemia (aCML) [1].

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Evaluation and Differential Diagnosis

Myelodysplastic syndromes are characterized by abnormal hematopoietic cell morphology – "dysplasia" – in at least 10% of cells in at least one cell line (erythroid, granulocytic, or megakaryocytic) with consequent decrease in one or more peripheral cell counts (red or white blood cells or platelets). Most commonly, MDS affects the red blood cell counts, causing normocytic or macrocytic anemia in approximately 75% of MDS patients. In addition, laboratory findings may include reticulocytopenia, neutropenia, thrombocytopenia, and/or circulating blasts.

The initial evaluation focuses on ruling out other common causes of anemia or pancytopenia, in addition to ruling out other etiologies that may mimic MDS. This workup includes a complete blood count with a differential leukocyte count, peripheral blood smear, iron studies (blood iron level, total iron-binding capacity, ferritin), reticulocyte count, and serum erythropoietin level. Vitamin deficiencies that can mimic MDS include folate deficiency (causing megaloblastic anemia), vitamin B12 deficiency (causing megaloblastic anemia and pancytopenia), and vitamin B6 deficiency (leading to siderblastic anemia). In addition, copper deficiency or excessive zinc intake that ultimately leads to copper deficiency can mimic MDS by causing bi-cytopenias (anemia and neutropenia) with the presence of ring sideroblasts and other dysplastic changes on bone marrow examination [16, 34]. Other differential diagnoses include drug-related morphologic abnormalities (methotrexate, valproate, mycophenolate mofetil), toxin-related effects (excessive alcohol use), autoimmune or thyroid disorders, or serious infections such as HIV. Once more common causes of cytopenias have been excluded, a bone marrow analysis should be obtained, without which a diagnosis of MDS is impossible [31].

Pathologic Features of MDS

The peripheral blood smear can show dysplastic changes in one or more cell lines. Red blood cells may have macrocytosis, anisocytosis, or a dimorphic pattern. Neutrophil dysplasia is typified by the presence of decreased or absent secondary granules, with some neutrophils showing nuclear hyposegmentation (pseudo Pelger-Huët anomaly), in which two nuclear masses (bilobed nucleus) are connected with a thin chromatin filament giving rise to the classic "pince-nez" nuclei. Platelets may be large or have decreased granules. The bone marrow in MDS is most commonly hypercellular. Dysplastic changes can affect one or more cell lines. The presence of increased blast cell percentages and Auer rods is variable. Dyserythropoiesis (dysplasia in the erythroid lineage) is associated with nuclear abnormalities (lobulation, irregular nuclear contours, and karyorrhexis). Ring sideroblasts (erythroblasts with iron-loaded mitochondria visualized by Prussian blue staining) can also be seen. Dysgranulopoiesis (dysplasia in the neutrophil myeloid cells) changes are similar to that seen in the peripheral blood, with cytoplasmic hypogranulation and nuclear hyposegmentation. Dysmegakaryopoiesis (dysplasia in the megakaryocytic lineage) can present as micromegakaryocytes with hypolobated nuclei. The bone marrow examination may reveal an increased myeloblast percentage of ≥5%. The presence of Auer rods on bone marrow evaluation carries a poorer prognosis and is seen in higher-risk MDS [2, 5].

Classification of Myelodysplastic Syndromes

French-American-British (FAB) Classification

In 1982 the FAB divided myelodysplastic syndromes into five categories: refractory anemia (RA), RA with ringed sideroblasts (RARS), RA with excess of blasts (RAEB, defined as <5% peripheral blood (PB) or 5–19% bone marrow (BM) myeloblasts), chronic myelomonocytic leukemia (CMML), and RAEB in transformation (RAEB-T, defined as ≥5% PB or 20–29% BM myeloblasts or presence of Auer rods). MDS was classified based on the percentage of bone marrow or peripheral blood blasts, peripheral monocytosis (>1000/μl for CMML), and the presence of >15% ringed sideroblasts (for RARS). However, this classification did not address the number of cell lines involved and the degree of dysplastic changes [2].

World Health Organization (WHO) Classification

The initial WHO classification was published in 2001 as part of the third edition of the "WHO Classification of Tumors." Major changes from the FAB classification included the percentage of bone marrow blasts to define acute myeloid leukemia (AML) decreasing from 30% to 20%, thus eliminating the RAEB-T classification. RAEB is divided into two subtypes: RAEB-1 (<5% PB and 5–9% BM blasts) and RAEB-2 (5–19% PB or 10–19% BM blasts). CMML was removed from the MDS classification and reclassified as an MDS/MPN overlap syndrome. In addition, a new entity, "MDS with isolated deletion 5q syndrome (del 5q)," was added to the classification, for the first time recognizing the importance of cytogenetic abnormalities in MDS [38].

In 2008, the fourth edition of the WHO classification was released. MDS was reclassified into seven entities depending on the number of peripheral cytopenias and number of dysplastic cell lines on bone marrow examination. These include refractory cytopenia with unilineage dysplasia (RCUD –

refractory anemia, refractory neutropenia, and refractory thrombocytopenia), refractory anemia with ring sideroblasts (RARS), refractory cytopenia with multilineage dysplasia (RCMD, involving two or more cell lines), RAEB-1 and RAEB-2, MDS with isolated del(5q), and MDS unclassifiable (MDS-U, which allows for specific recurring chromosomal abnormalities providing presumptive evidence of the diagnosis in the setting of persistent cytopenias without the presence of morphologic evidence of dysplasia) (see cytogenetic abnormalities, next section) (Table 25.1) [37].

The 2016 revision of the WHO classification, similar to previous classifications, focused on incorporating clinical, morphological, immunophenotypic, cytogenetic, and molecular features in diagnosing MDS. Major changes involved reduced emphasis on "cytopenias" and more on the degree of dysplasia and blast percentage, as it was believed that the degree of dysplasia often does not correlate with the specific cytopenia. The threshold to define dysplasia was kept at >10% dysplastic cells in any cell line. The new classification includes the following entities: MDS with single lineage dysplasia (MDS-SLD), MDS with ring sideroblasts (MDS-RS) which is further subdivided into single vs multilineage dysplasia (MDS-RS-SLD and MDS-RS-MLD), MDS with multilineage dysplasia (MDS-MLD), MDS with excess blasts (MDS-EB-1 and MDS-EB-2), MDS with isolated del(5q), and MDS unclassifiable (MDS-U). Refractory cytopenia of childhood was added to the classification as a new entity (Table 27.1) [1].

Cytogenetic and Molecular Characteristics

Clonal cytogenetic (chromosomal) abnormalities are detected in about 50% of de novo MDS cases and in 80% of patients with secondary MDS due to exposure to chemotherapy/ionizing radiation or toxins [12]. Several of these cytogenetic abnormalities are recurrent and characteristic of MDS. These typical karyotypic abnormalities allow a presumptive diagnosis of MDS solely based on their presence in the setting of persistent cytopenias, despite the absence of definitive morphological features. These include unbalanced chromosomal deletions of the long arm of chromosomes 5, 7, 9, 11, 13 or deletion of the short arm of chromosome 12 [del(12p)] or an unbalanced translocation in the short arm of chromosome 17 [t(17p)]. Other chromosomal abnormalities include balanced chromosomal translocations [t(11;16), t(3;21), t(1;3), t(2;11), t(6;9)] or inversion of chromosome 3 [inv(3)] [37]. Although trisomy 8, del(20q), and loss of Y chromosome (-Y) are common in MDS patients, they have been reported in other diseases and are thus not considered a sine qua non of MDS [21, 32]. Therapy-related MDS, occurring after exposure to chemotherapy or radiation therapy, commonly leads to abnormalities in chromosomes 5 or 7. Patients who harbor three or more cytogenetic abnormalities are classified as having a complex karyotype, conveying a poor risk.

Recurring genetic mutations can be broadly divided, based on their cellular function, into epigenetic regulators (TET2, ASXL1, EZH2, IDH1, IDH2, DNMT3A), DNA methylation, histone modification, mRNA-splicing factors (SF3B1, SRSF2, U2AF1), transcription factors (RUNX1 and TP53), and cytokine signaling pathway genes [27]. TP53 and RUNX1 mutations are more common in therapy-related MDS and are associated with resistance to treatment and poor outcomes [14].

International Prognostic Scoring Systems (IPSS and Revised IPSS)

The international prognostic scoring system is the most commonly used prognostication tool in MDS. The IPSS was devised to assess prognosis of primary untreated adults with MDS and was published in 1997 based on 816 patients with primary MDS. Patients were categorized according to the number of peripheral cytopenias, percentage of bone marrow blasts, and cytogenetic abnormalities. The threshold for defining a peripheral cytopenia was a hemoglobin <10 g/dL, absolute neutrophil count <1800/μL, and platelet count <100,000/μL (Table 27.2a). These values continue to be used in the 2016 WHO criteria to define cytopenias. The bone marrow blast percentage was divided into four groups (<5%, 5–10%, 11–20%, and 21–30%), acknowledging that blasts ≥20% defined acute leukemia. The number of cytogenetic or chromosomal abnormalities was categorized into good risk (normal, -Y, -5q, and -20q), intermediate risk, and poor risk (complex karyotype; i.e., ≥3 chromosomal abnormalities or chromosome 7 abnormalities) (Table 27.3). Based on the total IPSS score, patients are then classified into four risk groups (low, intermediate-1 ("lower risk"), intermediate-2, and high ("higher risk")). These risk categories aid in estimating patient median overall survival (5.7, 3.5, 1.2, and 0.4 years from diagnosis, respectively) and risk of progression into AML (Table 27.2b). In addition, treatment recommendations are different for patients with lower-risk MDS than higher-risk MDS [10].

The IPSS was revised (IPSS-R) in 2012 to include a larger cohort of 7012 patients with MDS. The number of peripheral cytopenias, bone marrow blast percentage, and cytogenetic/karyotype risk groups continued to form the basis of the classification. Additional factors that were noted to affect patient prognosis were serum lactate dehydrogenase, ferritin, and beta-2 microglobulin levels. The IPSS-R has five different cytogenetic risk groups (very good, good, intermediate, poor, and very poor), compared to three in IPSS, with the greatest weight now placed on cytogenetic risk (Table 27.3). The

Table 27.1 WHO classification of myelodysplastic syndromes

WHO 2016	Peripheral blood (PB)	Bone marrow (BM)	Cytogenetics	WHO 2008	Peripheral blood	Bone marrow
MDS with single lineage dysplasia (MDS-SLD)	<1% blasts 1 or 2 cytopenias[a]	<5% blasts	Any, unless fulfills all criteria for MDS with isolated del5(q)	Refractory cytopenia with unilineage dysplasia (RCUD)	Unicytopenia or bicytopenia[e]	Unilineage dysplasia: ≥10% of the cells in one myeloid lineage
		No Auer rods			No or rare blasts <1%	<5% blasts
		1 dysplastic lineage				<15% of erythroid precursors are ring sideroblasts
		Ring sideroblasts (RS) <15% or <5%[b]				
MDS with multilineage dysplasia (MLD-MLD)	<1% blasts 1–3 cytopenias	<5% blasts	Any, unless fulfills all criteria for MDS with isolated del5(q)	Refractory cytopenia with multilineage dysplasia (RCMD)	Cytopenia(s)	Dysplasia in ≥10% of the cells in ≥2 myeloid lineages (neutrophil and/or erythroid precursors and/or megakaryocytes)
		No Auer rods			No or rare blasts (<1%)[f]	<5% blasts
		2 or 3 dysplastic lineages			No Auer rods	No Auer rods
		RS <15% or <5%[b]				±15% ring sideroblasts
MDS with ring sideroblasts (MDS-RS) with single lineage dysplasia (MDS-RS- SLD)	<1% blasts 1 or 2 cytopenias	<5% blasts	Any, unless fulfills all criteria for MDS with isolated del(5q)	Refractory anemia with ring sideroblasts (RARS)	Anemia No blasts	≥15% of erythroid precursors are ring sideroblasts
		No Auer rods				Erythroid dysplasia only
		1 dysplastic lineage				<5% blasts
		RS ≥15% or ≥5%[b]				
MDS-RS with multilineage dysplasia (MDS-RS-MLD)	<1% blasts 1–3 cytopenias	<5% blasts	Any, unless fulfills all criteria for MDS with isolated del(5q)			
		No Auer rods				
		2 or 3 dysplastic lineages				
		RS ≥15% or ≥5%[b]				
MDS with isolated del(5q)	<1% blasts 1–2 cytopenias	<5% blasts	del(5q) alone or with 1 additional abnormality except −7 or del(7q)	MDS with isolated del(5q)	Anemia	Normal to increased megakaryocytes with hypolobulated nuclei
		No Auer rods			Usually normal or increased platelet count	<5% blasts
		1–3 dysplastic lineages			No or rare blasts (<1%)	Isolated del(5q) cytogenetic abnormality
		RS none or any				No Auer rods
MDS with excess blasts 1 (MDS-EB 1)	2–4% blasts 1–3 cytopenias	5–9% blasts	Any	Refractory anemia with excess blasts 1 (RAEB-1)	Cytopenia (s)	Unilineage or multilineage dysplasia
		No Auer rods			<5% blasts[f]	5–9% blasts[f]
		0–3 dysplastic lineages			No Auer rods	No Auer rods
		RS none or any			$<1 \times 10^9$/L monocytes	
MDS with excess blasts 2 (MDS-EB 2)	5–19% blasts Or Auer rods 1–3 cytopenias	10–19% blasts	Any	Refractory anemia with excess blasts 2 (RAEB-2)	Cytopenia (s)	Unilineage or multilineage dysplasia
		OR + Auer rods			5–19% blasts[g]	10–19% blasts[g]
		0–3 dysplastic lineages			± Auer rods[g]	± Auer rods[g]
		RS none or any			$<1 \times 10^9$/L monocytes	

MDS, unclassifiable (MDS-U) with 1% blood blasts	1% blasts[c] 1–3 cytopenias	<5% blasts	Any	MDS unclassified (MDS-U)	Cytopenias	Unequivocal dysplasia in <10% of cells in one or more myeloid lineages when accompanied by a cytogenetic abnormality considered as presumptive evidence for a diagnosis of MDS
		No Auer rods			≤1% blasts[f]	<5% blasts
		1–3 dysplastic lineages				
		RS none or any				
MDS-U with SLD and pancytopenia	<1% blasts 3 cytopenias	<5% blasts	Any			
		No Auer rods				
		1 dysplastic lineage				
MDS-U based on defining cytogenetic abnormality	<1% blasts 1–3 cytopenias	<5% blasts	MDS – defining abnormality			
		No Auer rods/dysplasia				
		RS <15% §				
Refractory cytopenia of childhood	<2% blasts 1–3 cytopenias	<5% blasts	Any	Provisional entity	N/A	N/A
		1–3 dysplastic lineages				

Adapted from Arber et al. [1] and Vardiman et al. [37]

WHO 2016 classification

[a]Cytopenias defined as hemoglobin <10 g/dl, platelet count $<100 \times 10^9$/L, and absolute neutrophil count $<1.8 \times 10^9$/L. Rarely MDS may present with mild anemia or thrombocytopenia above these levels. Peripheral blood monocytes must be $<1 \times 10^9$/L

[b]If SF3B1 mutation is present

[c]One percent PB blasts must be recorded on at least two separate occasions

[d]Cases with ≥15% ring sideroblasts by definition have significant erythroid dysplasia and are classified as MDS-RS-SLD

WHO 2008 classification

[e]Bicytopenia may occasionally be observed. Cases with pancytopenia were classified as MDS-U

[f]If the marrow myeloblasts percentage is <5% but there are 2–4% myeloblasts in the blood, the diagnostic classification is RAEB-1. Cases of RCUD and RCMD with 1% myeloblasts in the blood should be classified as MDS-U

[g]Cases with Auer rods and <5% myeloblasts in the blood and less than 10% in the marrow should be classified as RAEB-2. Although the finding of 5–19% blasts is, in itself, diagnostic of RAEB-2, cases of RAEB-2 may have <5% blasts in the blood if they have Auer rods or 10–19% blasts in the bone marrow or both. Similarly, cases of RAEB-2 may have <10% blasts in the marrow but may be diagnosed by the other two findings, positive Auer rods and/or 5–19% blasts in peripheral blood

Table 27.2 International prognostic scoring system (IPSS) classification

a					
	Score				
	0	**0.5**	**1.0**	**1.5**	**2.0**
Bone marrow blasts (%)	<5	5–10	–	11–20	21–30
Number of Cytopenias	0/1	2/3			
Karyotype[a]	Good	Intermediate	Poor		
b					
IPSS risk category	**Total IPSS score**	**Median overall survival (years)**		**25% AML evolution[b]**	
Low	0	5.7		9.4	
Intermediate-1	0.5–1	3.5		3.3	
Intermediate-2	1.5–2.0	1.2		1.1	
High	2.5–3.5	0.4		0.2	

Adapted from Greenberg et al. [10]
[a]Karyotype definitions: see Table 27.3
[b]Median time in years to evolution from MDS to AML in 25% of patients

Table 27.3 Cytogenetic scoring system

Per international prognostic scoring system (IPSS)		Per revised IPSS (IPSS-R)	
Cytogenetic risk group	Cytogenetic abnormality	Cytogenetic risk group	Cytogenetic abnormality
Good	Normal; del(5q); del(20q); -Y	Very good	-Y, del(11q)
		Good	Normal, del(5q), del(12p), del(20q), double including del(5q)
Intermediate	All others	Intermediate	del(7q), +8, +19, i(17q), any other single- or double-independent clones not including del(5q) or -7/del(7q)
Poor	Complex (≥3 abnormalities) or chromosome 7 abnormalities	Poor	-7, inv(3)/t(3q)/ del(3q), double including -7/ del(7q), complex: 3 abnormalities
		Very poor	Complex: >3 abnormalities

Adapted from Greenberg et al. [10, 11]

bone marrow blasts remained divided into four categories, with cut points for blast percentages altered. The revised classification is also more sensitive to degrees of cytopenias. As a result, the total risk score (0–10) categorizes patients into five prognostic risk groups (very low, low, intermediate, high, and very high) (Table 27.4a). The median overall survival in years from diagnosis and the risk of 25% of patients progressing into AML for the IPSS-R are summarized in Table 27.4b [11]. In estimating prognosis, it is important to also recognize that MDS patients are typically older adults; as such, mortality can often occur due to other medical comorbidities. Excess mortality in MDS has been shown to be related to older age, male sex, more comorbidities, and higher IPSS-R risk categories [22].

Treatment of Myelodysplastic Syndromes

Therapy for MDS is based largely on peripheral blood counts and whether patients have been determined to have lower- or higher-risk disease according to IPSS and IPSS-R risk scores. The international working group has devised response criteria of complete and partial responses and hematologic improvement in hemoglobin, platelet count, and/or absolute neutrophil count, which should be maintained for least 8 weeks. Treatment goals for lower-risk MDS center on minimizing transfusions and maximizing quality of life, while those for higher-risk disease should minimize transformation to AML and prolong survival [4].

Treatment of Lower Risk MDS

Lower-risk MDS falls into IPSS categories of low and intermediate-1, which largely correspond to IPSS-R risk groups very low, low, and some intermediate (IPSS-R score ≤3.5). Therapy is geared toward the predominating cytopenia, which for most lower-risk patients is anemia. Patients with lower-risk MDS with anemia respond well to erythropoiesis-stimulating agents (erythropoietin alpha and darbepoetin, with overall response rates of approximately 40%), provided that erythropoietin level at diagnosis is <500 U/L and transfusion needs are minimal. Responses should be assessed after 3 months of treatment, and duration of response can be up to 2 years. Patients with lower-risk MDS with del(5q) and anemia have a high transfusion independence response to lenalidomide (67%), which is FDA approved for that indication. Presence of a TP53 mutation was associated with poor response in del(5q) patients treated with lenalidomide. In the group of lower-risk patients without del(5q), transfusion-independence response rates are closer to 27%. For patients with lower-risk MDS and multiple cytopenias, the hypomethylating agents (azacitidine and decitabine) have an overall response rate of 30–40%. Immunosuppressive therapy with antithymocyte globulin with or without cyclosporine can also yield a response in 30–40% of patients. Bone marrow transplant remains the only curative treatment option for MDS patients but is reserved for lower-risk MDS patients who progress following other therapies and are at high risk of AML transformation (Fig. 27.1). The role of iron chelation therapy in MDS is unclear and largely discouraged [8, 18, 25, 30].

Table 27.4 Revised international prognostic scoring system (IPSS-R) classification

a							
	Score						
	0	**0.5**	**1.0**	**1.5**	**2.0**	**3.0**	**4.0**
Karyotype[a]	Very good	–	Good	–	Intermediate	Poor	Very poor
Bone marrow blasts (%)	≤2	–	>2 - <5	–	5–10	>10	
Hemoglobin (g/dl)	≥10		8 – <10	<8			
Absolute neutrophil count (cells/μl)	≥0.8	<0.8					
Platelets (cells/μl)	≥100	50–100	<50				

b			
IPSS-R risk category	**Total IPSS-R score**	**Median overall survival (years)**	**25% AML evolution[b]**
Very low	≤1.5	8.8	Not reached
Low	>1.5–3	5.3	10.8
Intermediate	>3–4.5	3.0	3.2
High	>4.5–6	1.6	1.4
Very high	>6	0.8	0.7

Adapted from Greenberg et al. [11]
[a]Karyotype definitions: see Table 27.3
[b]Median time in years to evolution from MDS to AML in 25% of patients

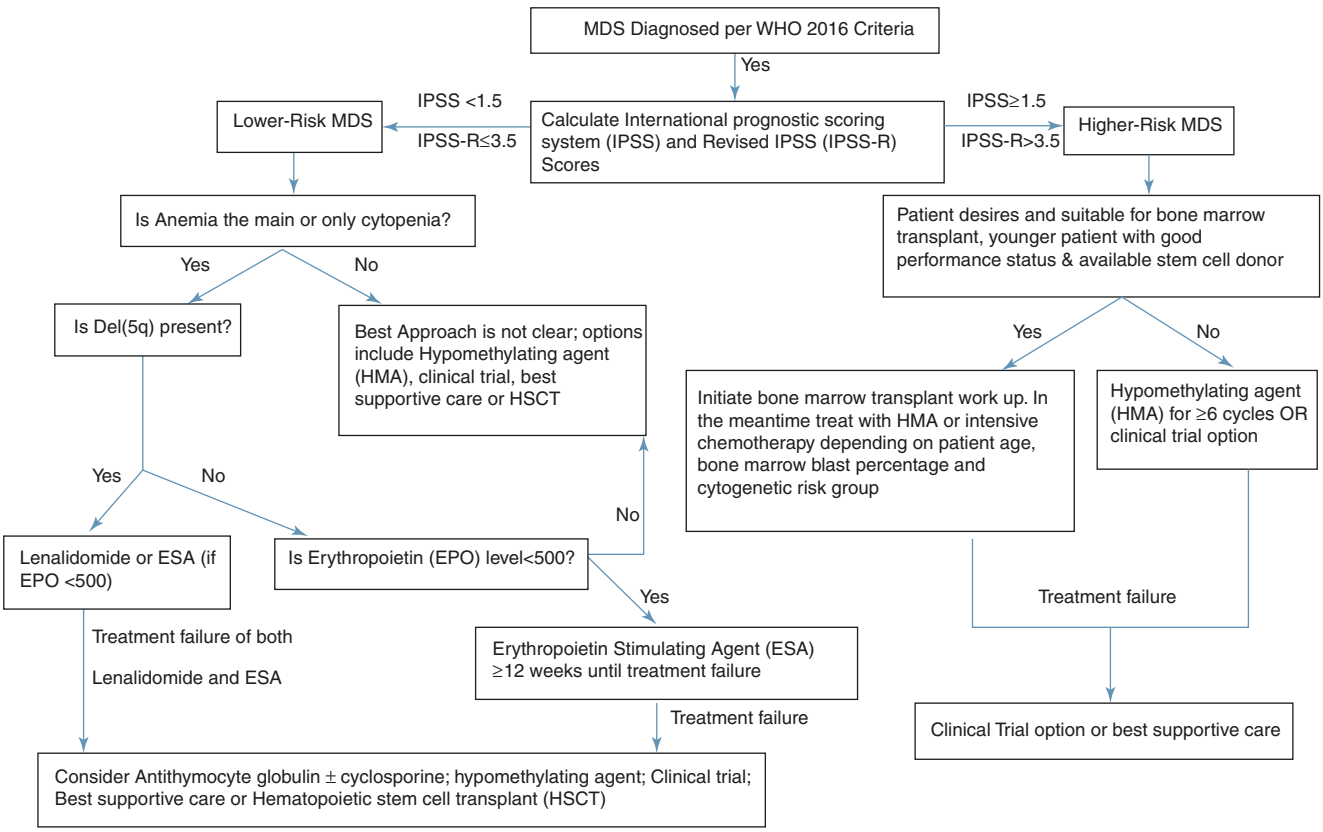

Fig. 27.1 Treatment algorithm for myelodysplastic syndromes

Treatment of Higher Risk MDS

Patients with higher-risk MDS fall into IPSS risk categories of intermediate-2 and high risk (IPSS score ≥1.5), corresponding to IPSS-R risk groups high and very high and some intermediate (IPSS-R score >3.5). Patients with MDS with excess blasts -1 (MDS-EB-1) and MDS-EB-2 according the 2016 WHO classification are also sometimes included in the higher-risk group. Patients with higher-risk MDS should be treated with a hypomethylating agent (azacitidine or decitabine), both of which are FDA approved for this indication. In the only phase 3 clinical trial to show a survival advantage for drug therapy in

MDS, higher-risk patients receiving azacitidine had an overall response rate of 35% and a median overall survival of 24.5 months, compared to 15.0 months for patients randomized to conventional care regimens (best supportive care, low-dose cytarabine, or cytarabine + daunorubicin, $p < 0.001$). A phase 3 trial of decitabine vs. best supportive care showed higher overall response rates for decitabine vs. best supportive care but no difference in overall survival (10.1 months vs. 8.5 months, $p = 0.38$) [9, 19]. Mutations in methylation genes appear to be associated with better response to these agents [36]. Treatment with hypomethylating agents should be continued for at least 6 months prior to determining lack of efficacy and should be continued as long as a response is maintained. The possibility of hematopoietic cell transplantation should be assessed and discussed with every patient at diagnosis of higher-risk MDS, as this is the only potentially curative option (Fig. 27.1) [13, 27]. Unfortunately, after failure of hypomethylating agent therapy, treatment options are limited. Multiple clinical trials are ongoing to assess the response to novel agents, immune modulation, and trying different combination therapies [3].

Summary

Myelodysplastic syndromes are a group of heterogeneous, clonal, bone marrow disorders associated with cytopenias. Specific chromosomal and molecular abnormalities drive the disease pathogenesis. MDS classification is based on these features, as are prognostic scoring systems, which represent default staging systems and impact treatment decisions. Lower-risk MDS is treated to improve patient symptoms, minimize transfusions, and improve quality of life, whereas the goal of treating higher-risk MDS is to delay AML transformation and improve overall survival. Hematopoietic cell transplantation remains the only curative option, while non-transplant therapies can extend survival.

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Acute Leukemia

28

Kamal Menghrajani and Martin S. Tallman

Leukemias are an uncommon and heterogeneous group of diseases characterized by infiltration of the bone marrow, blood, and visceral organs by neoplastic cells of the hematopoietic system. Leukemias generally stem from the myeloid or lymphoid hematopoietic lineages and occur as acute or chronic disease. This chapter will focus on the acute leukemias, namely, acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL). Acute leukemia is diagnosed by the discovery of ≥20% blasts in the peripheral blood or bone marrow, although such stringent cutoffs may not accurately define the biology of the disease.

Advances in the understanding of the molecular drivers of these diseases have led to further classification into subgroups, which can give prognostic information and, in some cases, may allow for the use of targeted treatment approaches. This classification is based on integrated results of morphology, immunohistochemistry, immunophenotyping by flow cytometry, cytogenetics (karyotyping), FISH, and molecular studies of mutations shown in Fig. 28.1.

Acute Myeloid Leukemia

AML, arising from hematopoietic precursors of the nonlymphoid compartment, is the most common acute leukemia in adults, with an annual incidence of 4.3/100,000. The frequency increases with age, with a median age at diagnosis of 70 years. Approximately 2.8/100,000 people are expected to die of AML each year, and only 27.4% of patients are alive at 5 years after diagnosis.

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Clinical Features, Workup, and Diagnosis

Patients with AML commonly present with symptoms that reflect underlying bone marrow failure. This causes symptoms reflecting anemia, such as pallor, fatigue, weakness, palpitations, or dyspnea on exertion. Symptoms of thrombocytopenia, including ecchymoses, petechiae, epistaxis, and prolonged bleeding after minor injury may also be seen. While patients may be found to be neutropenic, major infections are uncommon at diagnosis. Patients may also have systemic symptoms, including anorexia, weight loss, and low-grade fevers. Hepatomegaly or splenomegaly may be seen in approximately one-third of patients.

The initial evaluation of AML should focus on both the characteristics of the disease and the comorbidities of the patient. A bone marrow biopsy and aspiration should be performed to evaluate the blast percentage (see Fig. 28.2 for a representative marrow). Additionally, the disease should be evaluated on factors including cytogenetic or molecular abnormalities, antecedent myelodysplasia, or prior exposure to cytotoxic chemotherapy, as such an assessment offers prognostic information and may change therapy.

Some patients may present with disseminated intravascular coagulation (DIC), especially those with acute promyelocytic leukemia. As such, evaluation of the platelet count, prothrombin time, activated partial thromboplastin time, and serum fibrinogen should be part of the initial evaluation.

Hyperleukocytosis, i.e., the presentation of a high white blood cell count usually greater than $>100 \times 10^9$/L, is seen in approximately 5% of patients. Hyperleukocytosis can lead to leukostasis, a symptomatic rise in the blast count which leads to decreased tissue perfusion, due to increases in blood viscosity and cytokine release from high cellular metabolic activity. Leukostasis is a medical emergency and can manifest with CNS, pulmonary, or other symptoms due to leukostatic plugs preventing adequate tissue oxygenation. CNS manifestations include blurred vision, papilledema, retinal hemorrhages, dizziness, slurred speech, stupor, delirium, or intracranial hemorrhage. Pulmonary symptoms may include tachypnea, hypoxia,

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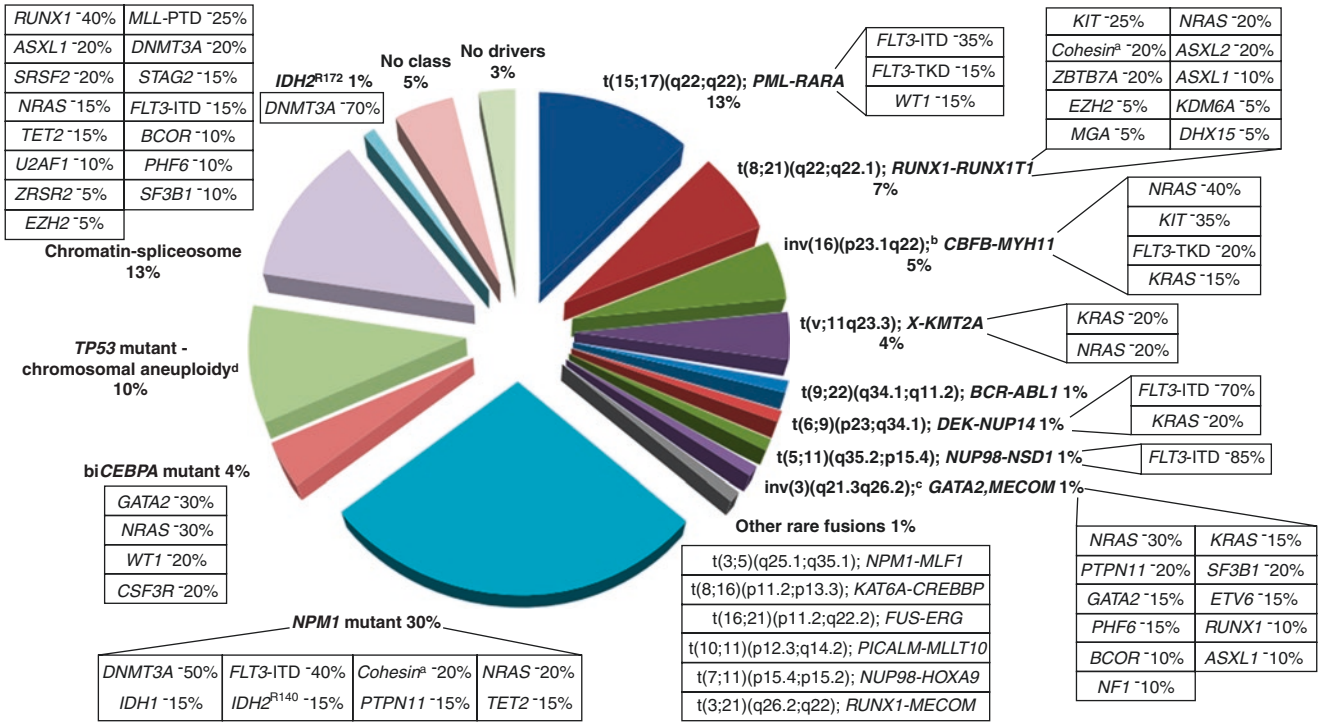

Fig. 28.1 Frequency of prognostically relevant molecular and cytogenetic subgroups of AML from the 2017 European Leukemia Network (ELN) guidelines [4]

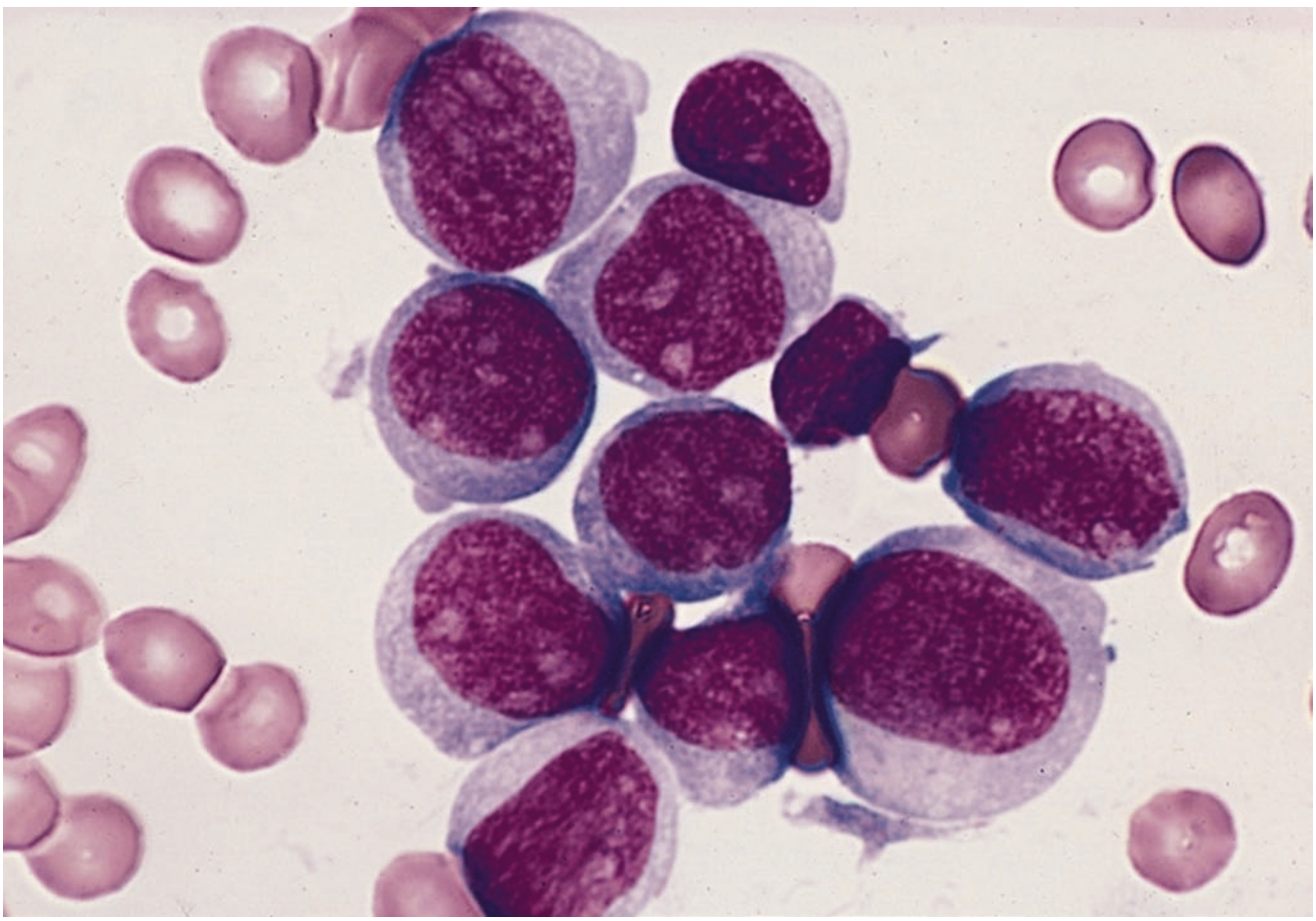

Fig. 28.2 Myeloid blasts in bone marrow. (Used from The Armed Forces Institute of Pathology)

cyanosis, dyspnea, and pulmonary infiltrates. When both neurologic manifestations and respiratory failure are present, patients have a predicted mortality rate of 90% by 1 week from diagnosis. Male patients may also experience priapism.

Leukostasis is acutely treated by lowering the white blood cell count (cytoreduction). This can be done with hydroxyurea, an antimetabolite which can drop the WBC by up to 80% within 24–48 h. As leukostasis is a medical emergency, hydroxyurea is commonly reserved for asymptomatic patients with hyperleukocytosis, and a more rapid approach may be chosen for those with symptomatic leukostasis. Such a rapid approach may include initiation of induction chemotherapy, with the understanding that this may induce rapid cellular lysis and precipitate tumor lysis syndrome. Induction chemotherapy may decrease the WBC count by 24 h and may be an effective strategy for rapid cytoreduction. Mechanical removal of excess WBCs can also be performed using a technique known as leukapheresis. Leukapheresis involves the placement of a large-bore central venous catheter, such as a dialysis catheter, that will allow for the withdrawal of blasts from the circulation. The effects of leukapheresis on mortality are controversial, and several sessions may be required to improve patient symptoms and lower the WBC. Given the risks involved in catheter placement and the lack of directed treatment at the underlying cause, leukapheresis should be used as an adjunct or a bridge to leukemia-directed therapy.

While CNS disease is less common with AML than it is with ALL, certain patients are at higher risk. Those with symptoms suggestive of CNS disease, monocytic differentiation, a high white blood cell count (>40,000/μL) at presentation, extramedullary disease, high-risk acute promyelocytic leukemia, or mixed-phenotype acute leukemia should undergo a lumbar puncture at first remission.

For patients who present with extramedullary disease without bone marrow involvement, commonly called myeloid sarcoma, PET/CT should be performed to evaluate for additional sites of disease.

Initial Evaluation for AML

- Thorough history and physical exam, including evaluation of performance status
- CBC with platelets and differential
- Peripheral smear evaluation
- Serum chemistries and liver function tests
- Disseminated intravascular coagulation (DIC) panel (D-dimer, fibrinogen, PT/aPTT, platelets)
- Tumor lysis syndrome (TLS) labs (serum lactate dehydrogenase (LDH), uric acid, potassium, phosphate, calcium)
- Bone marrow analysis with aspirate and morphology review, cytogenetics/FISH, flow cytometry, myeloid mutation panel, Rapid IDH1, IDH2, and FLT3 mutational analysis
- Urinalysis
- Hepatitis B/C, HIV, CMV antibody testing
- Pregnancy testing in females
- Fertility counseling
- Evaluation of cardiac function (e.g., transthoracic echocardiogram)
- Head imaging and LP if concern for CNS involvement
- PET/CT if concern for CNS or other extramedullary involvement

Classification

The classification of AML has evolved from the previous French-American-British (FAB) system, which was based on morphology alone, to the World Health Organization system, which incorporates cytogenetics, immunophenotypic analysis, and molecular abnormalities.

In this schema, AML is diagnosed with presence of 20% blasts detected in the peripheral blood or bone marrow. The WHO does identify certain clonal cytogenetic abnormalities, namely, t(15;17), t(8;21), and inv(16), whose presence should be considered diagnostic of AML, regardless of the percentage of blasts detected.

Details of risk stratification by the 2016 WHO classification schema are presented in Table 28.1

Risk Stratification

Cytogenetics are the most important prognostic factor in predicting rate of remission, risk of relapse, and overall survival outcomes. Patients with t(8;21) and inv(16) without a *c-kit*

Table 28.1 WHO classification of myeloid neoplasms and acute leukemia [6]

WHO Myeloid Neoplasom and Acute Leukemia Classification
Myeloproliferative neoplasms (MPN)
Chronic myeloid leukemia (CML). *BCR-ABL1*$^{+}$
Chronic neutrophilic leukemia (CNL)
Polycythemia vera (PV)
Primary myelofibrosis (PMF)
PMF, prefibrotic/early stage
PMF, overt fibrotic stage
Essential thrombocythemia (ET)
Chronic eosinophilic leukemia, not otherwise specified (NOS)
MPN, unclassifiable
Mastocytosis

(continued)

Table 28.1 (continued)

WHO Myeloid Neoplasom and Acute Leukemia Classification
Myeloid/lymphoid neoplasms with eosinophilia and rearrangement of *PDGFRA*, *PDGFRS*, or *FGFR1*, or with *PCM1-JAk2*
Myeloid/lymphoid neoplasms with *PDGFRA* rearrangement
Myeloid/lymphoid neoplasms with *PDGFRB* rearrangement
Myeloid/lymphoid neoplasms with *FGFR1* rearrangement
Provisional entity: Myeloid/lymphoid neoplasms with PCM1-JAK2
Myelodysplastic/myeloproliferative neoplasms (MDS/MPN)
Chronic myelomonocytic leukemia (CMML)
Atypical chronic myeloid leukemia (aCML), *BCR-ABL1*$^{-}$
Juvenile myelomonocytic leukemia (JMML)
MDS/MPN with ring sideroblasts and thrombocytosis (MDS/MPN-RS-T)
MDS/MPN, unclassifiable
Myelodysplastic syndromes (MDS)
MDS with single lineage dysplasia
MDS with ring sideroblasts (MDS-RS)
MDS-RS and single lineage dysplasia
MDS-RS and multilineage dysplasia
MDS with multilineage dysplasia
MDS with excess blasts
MDS with isolated del(5q)
MDS, unclassifiable
Provisional entity: Refractory cytopenia of childhood
Myeloid neoplasms with germ line predisposition
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 I(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

Table 28.2 ELN risk stratification by genetics [4]

Risk category	Genetic abnormality
Favorable	t(8;21)(q22;q22.1); *RUNX1-RUNX1T1*
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
	Mutated *NPM1* without *FLT3-ITD* or with *FLT3*-ITD^{low}
	Biallelic mutated *CEBPA*
Intermediate	Mutated *NPM1* and *FLT3*-ITD^{high}
	Wild-type *NPM1* without *FLT3*-ITD or with *FLT3*-ITD^{low} (without adverse-risk genetic lesions)
	t(9;11)(p21.3;q23.3); *MLLT3-KMT2A*
	Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23;q34.1); *DEK-NUP214*
	t(v;11q23.3); *KMT2A* rearranged
	t(9;22)(q34.1;q11.2); *BCR-ABL1*
	inv(3)(q2l.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2,MECOM(EVI1)*
	−5 or del(5q); −7; −17/abn(17p)
	Complex karyotype, monosomal karyotype ll
	Wild-type *NPM1* and *FLT3*-ITD^{high}
	Mutated *RUNX1*
	Mutated *ASXL1*
	Mutated *TP53*

mutation and those with t(15;17) are thought to have favorable-risk disease, based on the 2017 European Leukemia Network (ELN) guidelines. Adverse-risk disease is seen in patients with cytogenetic abnormalities including complex karyotype (≥3 cytogenetic abnormalities), monosomal karyotype (the presence of a monosomy in addition to a second monosomy or other cytogenetic abnormality), and −5/del(5q); −7; t(6;9); t(v;11q23); t(9;22); inv(3). Intermediate-risk classification is applied to patients with t(9;11), trisomy 8, normal cytogenetics, or other cytogenetic changes not otherwise defined.

Molecular data are also being used to risk stratify patients, and this field is rapidly evolving. Based on the 2017 ELN guidelines, patients with mutated *NPM1* without *FLT3*-ITD and those with biallelic mutations in *CEBPA* are considered favorable risk. Those with wild-type *NPM1* and *FLT3*-ITD^{high} or with mutated *RUNX1*, *ASXL1*, or *TP53* are considered adverse-risk. With the advent of myeloid genomic testing being incorporated into the standard of care, further studies are likely to identify additional mutational profiles that are of prognostic significance (Table 28.2).

Treatment

Induction

The intent of induction chemotherapy is to achieve remission to gain initial control over the disease, as well as to restore

normal hematopoiesis. The choice of induction therapy is influenced by individual characteristics of both the patient and the disease.

Standard induction regimens utilize cytarabine in combination with an anthracycline. The anthracycline daunorubicin has been studied more commonly than idarubicin, although both have been found to have comparable remission rates. Mitoxantrone is an anthracenedione and has also been studied in combination with cytarabine for induction therapy but is more commonly used in pediatric regimens.

For patients 60 years of age or younger, cytarabine 100 mg/m^2 administered as a continuous IV infusion for 7 days in combination with daunorubicin 90 mg/m^2 daily for 3 days ("7 + 3") is a commonly used regimen. Daunorubicin 90 mg/m^2 was compared to 45 mg/m^2 in patients 60 years and younger and was shown to produce a higher rate of complete remissions and longer overall survival without a significant increase in cardiotoxicity.

For patients over 60 years of age, daunorubicin at a dose of 60 mg/m^2 in combination with cytarabine is considered a standard "7 + 3" regimen for those who are fit and wish to undergo induction therapy. Some European studies suggest that the addition of a purine analog (cladribine or fludarabine) to an induction regimen can improve overall survival; however, this has not been adopted as standard practice in the United States.

In recent years, induction chemotherapy regimens have begun to change for certain populations. For elderly adults who are unfit or do not wish to undergo treatment with intensive chemotherapy, initial therapy may include the combination of venetoclax with low-dose cytarabine. Patients who have a therapy-related myeloid neoplasm or who have AML with myelodysplasia-related changes may be candidates for treatment with liposomal daunorubicin and cytarabine, known as CPX-351. Finally, patients with *FLT3* mutations may benefit from the addition of midostaurin to their induction and consolidation regimens.

Consolidation

Post-remission therapy, known as consolidation, is also necessary – patients who do not receive post-induction therapy may relapse within 6–9 months. The choice of consolidation therapy may depend on the patient's risk stratification. Patients who have so-called favorable-risk disease may be treated with high-dose cytarabine alone, commonly given as 3 g/m^2 IV every 12 hours on days 1, 3, and 5 with G-CSF support, although bone marrow transplantation can be considered in certain cases. Those with intermediate- or adverse-risk disease may have a higher risk of relapse and should strongly consider consolidative treatment with an allogeneic hematopoietic cell transplant (HCT), with or without additional chemotherapy (such as high-dose cytarabine) given prior to HCT.

CNS Disease

Leptomeningeal disease is somewhat infrequent in AML, especially as compared to ALL, and occurs in <3% of AML cases. As such, lumbar puncture (LP) is not usually performed as part of the routine diagnostic workup. For patients who present with symptoms such as headache, confusion, altered sensorium, etc., initial head imaging (i.e., CT or MRI) should be performed to rule out intracranial hemorrhage or the presence of a mass lesion prior to an LP. If the imaging and LP are negative, patients can be clinically followed for symptoms with repeat evaluation as warranted by clinical status. If the LP is positive, IT chemotherapy should be given concurrently with standard induction. Generally, IT chemotherapy (e.g., with cytarabine, methotrexate, or a regimen that incorporates both) is given twice weekly until blasts are cleared from the CNS and then weekly for an additional 4–6-week period. The CNS should again be assessed in the post-induction setting with additional IT therapy given as appropriate.

For patients in whom imaging detects a mass lesion or parenchymal involvement, needle aspiration may be considered to confirm the diagnosis. If confirmed, the patient may be offered radiation therapy followed by IT chemotherapy. HIDAC, which penetrates the blood-brain barrier and can be used as both systemic and CNS-directed therapy or focused IT therapy, should not be given concurrently with cranial irradiation as this may increase neurotoxicity.

Response Criteria

As per the WHO [6], response to therapy is categorized as follows:

- Complete response (CR): ANC >1000/μL, platelets ≥100,000/μL, no residual evidence of extramedullary disease, and transfusion independence
- Complete response with incomplete count recovery (CR_i): <5% bone marrow blasts, transfusion independence but with a persistent cytopenia (either ANC <1000/μL or thrombocytopenia <100,000/μL.
- Cytogenetic CR – normal cytogenetics in those with previous abnormalities
- Molecular CR (APL and Ph+ ALL only) – qPCR negative
- Partial remission (PR): 50% decrease in the blast percentage, with a blast percentage of between 5% and 25%, not meeting criteria for any type of CR
- Relapse: Reappearance of leukemic blasts in the peripheral blood, the finding of >5% bone marrow blasts, or recurrence of extramedullary disease

Minimal Residual Disease

Minimal residual disease (MRD) is the presence of leukemic cells in the bone marrow after treatment which can be

detected by flow cytometry or molecular testing. The role of MRD in the prognosis and treatment of AML is still under study. Current data suggest that patients who are MRD positive after treatment are more likely to relapse, even after HCT. Studies are underway to determine what strategies may be effective in eradicating MRD.

Acute Promyelocytic Leukemia

APL is an aggressive subtype of AML with distinct morphological and clinical features. It is cytogenetically characterized by the presence of translocation t(15;17), which leads to the production of a *PML-RARα* fusion gene. The fusion transcript also is detected and monitored by qPCR to document disease burden, with a goal of achieving molecular remission with treatment.

Historically, APL has been associated with a high early death rate related to coagulopathy that is often seen at presentation. It has also been described as occurring as therapy-related disease after treatment with topoisomerase II inhibitors and/or radiation. Despite its aggressive nature, treatment of APL is now leading to cure rates of up to 99% without bone marrow transplantation.

Treatment for APL is chosen based on stratification by WBC, with ≤10,000 cells/μL considered low risk and levels above this threshold considered high risk. Low-risk disease is treated with induction therapy that incorporates all-trans retinoic acid (ATRA), which can induce differentiation of APL blasts and reverse the coagulopathy commonly seen with this disease. The most common regimen, published by LoCoco et al. in NEJM in 2013, uses ATRA and arsenic trioxide (ATO) for both induction and consolidation therapy, with long-term follow-up studies showing persistent response rates of as high as 99% in low-risk patients. Patients with high-risk disease are treated with a combination regimen that includes ATRA and an anthracycline, such as ATRA with daunorubicin and cytarabine or ATRA with idarubicin and ATO.

Patients who are thought to have APL based on morphology, presence of coagulopathy or disseminated intravascular coagulation, or immunophenotype should be started on ATRA without awaiting confirmation of the diagnosis. If APL is not confirmed by cytogenetics or FISH, ATRA can be discontinued in favor of alternative therapy; however, a delay in initiating therapy for a patient with APL may be fatal.

Treatment with ATRA may induce what is known as differentiation syndrome, heralded by fever, shortness of breath, hypoxemia, and pleural or pericardial effusions. Patients with high-risk disease (WBC >10,000/μL) should receive steroid prophylaxis, and patients who develop differentiation syndrome should be treated with dexamethasone 10 mg every 12 hours, and ATRA should be temporarily held. Treatment with ATO can cause QT prolongation, requiring EKG and electrolyte monitoring.

Acute Lymphoblastic Leukemia

Acute lymphoblastic leukemia, sometimes called acute lymphocytic leukemia, is a clonal, neoplastic disease of immature lymphocytes derived from either B- or T-cell lineage. ALL is the most common malignancy in childhood (3–4/100,000) with a peak incidence occurring between 2 and 3 years of age and most cases occurring before age 10. The incidence of adult ALL is much lower, at about 1.7/100,000 people annually in the United States (Fig. 28.3).

Clinical Features, Workup, and Diagnosis

As with acute myeloid leukemia, patients may present with nonspecific symptoms. Adults with ALL may sometimes initially complain of fatigue, B-symptoms (e.g., fevers, chills, night sweats, and weight loss), dyspnea, easy bruising, bleeding, or petechiae. Bone pain can be seen but is more common in children. Approximately 20% of patients may present with lymphadenopathy, splenomegaly, and possibly hepatomegaly. Patients who complain of perioral or chin numbness, headache, confusion, or cranial nerve abnormalities should be evaluated for leptomeningeal disease.

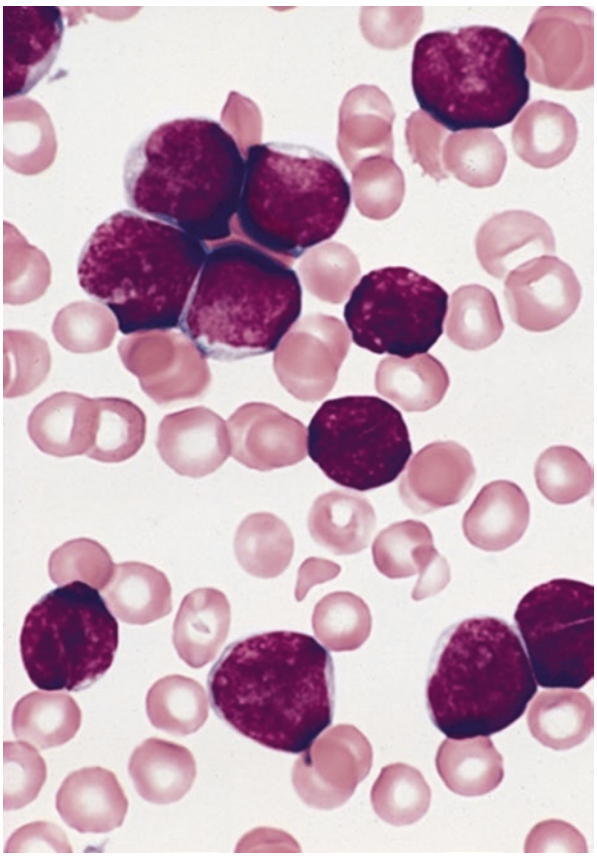

Fig. 28.3 Acute lymphoblastic leukemia. (Used from The Armed Forces Institute of Pathology)

Patients may present with an elevated white blood cell count, with a WBC >30,000/μL for B-lineage and >100,000/μL T-lineage disease being adverse prognostic factors, and evaluation of the peripheral blood smear may show circulating lymphoblasts. In cases with an elevated white blood cell count and symptoms including dyspnea, hypoxemia, confusion, visual changes, tinnitus, gait instability, somnolence, or fever, leukostasis should be considered – although this is less commonly seen than in AML. Some patients also present with thrombocytopenia, which may manifest as purpura, menorrhagia, mucosal bleeding, or even retinal hemorrhage.

Workup for the disease includes a history and physical exam, laboratory studies, imaging studies where appropriate, evaluation for opportunistic infections, and fertility counseling, and should be tailored to each individual case.

Initial Evaluation for ALL

- Thorough history and physical exam, including evaluation of performance status
- CBC with platelets and differential
- Peripheral smear evaluation
- Serum chemistries and liver function tests
- Disseminated intravascular coagulation (DIC) panel (D-dimer, fibrinogen, PT/aPTT, platelets)
- Tumor lysis syndrome (TLS) labs [serum lactate dehydrogenase (LDH), uric acid, potassium, phosphate, calcium]
- Bone marrow analysis with aspirate and morphology review, cytogenetics/FISH, flow cytometry, and gene fusion panel
- Urinalysis
- Hepatitis B/C, HIV, and CMV antibody testing
- Pregnancy testing in females
- Evaluation of testicular involvement in male patients
- Fertility counseling
- Evaluation of cardiac function (e.g., transthoracic echocardiogram)
- CNS imaging as appropriate
- Lumbar puncture as per treatment protocol (diagnostic LP commonly performed at time of first intrathecal treatment)

Acute lymphoblastic leukemia is considered to be the same entity as lymphoblastic lymphoma, as per the 2016 WHO guidelines. The distinction between the two is that the bone marrow is involved with 20% lymphoblasts or greater in acute lymphoblastic leukemia, whereas the disease is restricted to a mass lesion involving nodal or extranodal sites with minimal or no involvement in the blood or bone marrow in lymphoblastic lymphoma. As such, a bone marrow biopsy and aspiration is necessary to confirm the diagnosis.

Flow cytometry is also used to classify ALL into three immunophenotypic subgroups, namely, precursor B-cell ALL, mature B-cell ALL, and T-cell ALL. A representative photomicrograph of precursor B-cell ALL cells in the bone marrow is shown in Fig. 28.3. In addition, cytogenetic and molecular studies are often sent at diagnosis from a bone marrow sample to assist in risk stratification and choice of an optimal treatment regimen.

Immunophenotypic Characterization

In adults, approximately 75% of ALL cases evolve from B-cells. The immunophenotype of early precursor B-cell ALL is characterized by the presence of terminal deoxynucleotidyl transferase (TdT), the expression of CD19/CD22/CD79a, and the absence of CD10 or surface immunoglobulins.

The immunophenotype of precursor B-cell ALL is characterized by the presence of CD10, CD19, CD22, and CD79a, as well as the presence of cytoplasmic immunoglobulins.

Mature B-cell ALL has an immunophenotype that is positive for surface immunoglobulins and clonal lambda or kappa light chains and is negative for TdT.

CD20 expression can be seen in approximately 50% of adult B-cell ALL cases and is most commonly seen in the mature B-cell subtype.

T-cell ALL typically presents with an immunophenotype of cytoplasmic or cell surface CD3, in addition to variable expression of CD1a/C2/CD5/CD7 and expression of TdT. Around 30–50% of the cells may also express CD52.

Early T-cell precursor (ETP) ALL is seen in about 2% of adult ALL and was traditionally associated with poor clinical outcomes. ETP ALL is usually characterized by the absence of CD1a/CD8, weak expression of CD5, and expression of one or more myeloid or stem cell markers, including CD34, CD33, CD11b, CD117, HLA-DR, CD13, and CD65. ETP was previously thought to have a poorer prognosis, but more recent data suggest that outcomes for these patients may be no worse than for patients with other subtypes of ALL, if they are treated effectively.

A subset of acute leukemias are of ambiguous lineage and are considered to be "mixed phenotype" acute leukemias (MPAL). These leukemias have features of both AML and ALL and can be treated based on their molecular characteristics (e.g., TKIs can be used in the presence of a t(9;22)).

Chromosomal and Molecular Changes

Table 28.3 below outlines some of the most common chromosomal and molecular abnormalities seen in 2016 WHO classification of ALL.

Table 28.3 2016 WHO classification of ALL [6]

B-lymphoblastic leukemia/lymphoma
B-lymphoblastic leukemia/lymphoma, NOS
B-lymphoblastic leukemia/lymphoma with recurrent genetic abnormalities
B-lymphoblastic leukemia/lymphoma with t(9;22)(q34.1;q11.2);*BCR-ABL1*
B-lymphoblastic leukemia/lymphoma with t(v;11q23.3);*KMT2A* rearranged
B-lymphoblastic leukemia/lymphoma with t(12;21)(p13.2;q22.1); *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;p13.30);*TCF3-PBX1*
Provisional entity: B-lymphoblastic leukemia/lymphoma, BCR-ABL1-like
Provisional entity: B-lymphoblastic leukemia/lymphoma with iAMP21
T-lymphoblastic leukemia/lymphoma
Provisional entity: Early T-cell precursor lymphoblastic leukemia
Provisional entity: Natural kilter (NK) cell lymphoblastic leukemia/lymphoma

Numerical changes in chromosomes are common in this disease. Hypodiploidy (<46 chromosomes), pseudodiploidy (46 chromosomes with other structural abnormalities), and hyperdiploidy (>50 chromosomes) are frequently seen. Hyperdiploidy confers a favorable prognosis unless the patient is near triploidy; low hypodiploidy and pseudodiploidy/complex karyotype (≥5 cytogenetic abnormalities) notably both have a poor prognosis.

Table 28.4 (below) further describes the common chromosomal and molecular abnormalities seen in ALL. Translocation t(9;22), also called a Philadelphia chromosome, leads to a BCR-ABL1 gene fusion product and is seen commonly in B-cell ALL in adults, occurring in about 25% of cases. This gene fusion product encodes a tyrosine kinase which is constitutively active and interferes with signaling pathways such as RAS. Philadelphia chromosome positivity can indicate a poorer prognosis and can also affect the choice of therapy, as discussed below.

Detection of a Philadelphia chromosome-like phenotype, in which the gene expression profile is similar to that seen in Ph-positive B-ALL but without the presence of a translocation t(9;22), also carries an unfavorable prognosis – one study of adult patients found a 5-year event-free survival of 22.5%. Philadelphia-like ALL may involve a mutation of the Ikaros (*IKZF1*) gene, but mutations in genes such as *ABL1*, *ABL2*, *EPOR*, *JAK2*, *PDGFRβ*, *EBF1*, *FL2*, *IL7R*, *NTRK3*, and *SH2B3* have also been found to generate this phenotype.

The presence of a translocation t(12;21) defines an *ETV6-RUNX1* fusion, also called *TEL-AML1*. This is the most common genetic abnormality noted in childhood ALL and is associated with a favorable prognosis (see Chap. 35). Translocation t(v;11q23) involves the mixed-lineage leukemia gene (*MLL*) which encodes a lysine methyltransferase and is therefore also known as *KMT2A*; translocations at this site portend a poor prognosis. Finally, translocation t(8;14) is associated with a mature B-cell phenotype and dysregulation of the c-myc proto-oncogene; it occurs in about 5% of ALL and confers a poor prognosis.

Table 28.4 Common chromosomal and molecular abnormalities in ALL [2]

Cytogenetics	Gene	Frequency in adults	Frequency in children
Hyperdiploidy (>50 chromosomes)	–	7%	25%
Hypodiploidy (<44 chromosomes)	–	2%	1%
t(9;22)(q34;q11): Philadelphia chromosome (Ph)	*BCR-ABL1*	25%	2–4%
t(12;21)(p13;q22)	*ETV6-RUNX1(TEL-AML1)*	2%	22%
t(v;11q23)[e.g., t(4;11), t(9;11)], t(11;19)	*KMT2A (MLL)*	10%	8%
t(1;19)(q23;pl3)	*TCF3-PBX1(E2A-PBX1)*	3%	6%
t(5;14)(q31;q32)	*IL3-IGH*	<1%	<1%
t(8;14), t(2;8), t(8;22)	*c-MYC*	4%	2%
t(1;l4)(p32;q11)	*TAL-1*[a]	12%	7%
t(10;l4)(q24;q11)	*HOX11(TLX1)*[a]	8%	1%
t(5;14)(q35;q32)	*HOX11L2*[a]	1%	3%
t(11;l4)(q11) [e.g., (pl3;q11), (p15;q11)]	*TCRα and TCRδ*	20–25%	10–20%
BCR-ABL1-like	*Various*[b]	10–30%	15%
ETP	*Various*[a]	2%	2%
Ikaros	*IKZF1*	25–35%	12–17%

[a]Abnormalities observed exclusively in T-cell lineage ALL; all others occur exclusively or predominately in B-cell lineage ALL
[b]See text for more details

Early T-cell precursor (ETP) lymphoblastic leukemia was also previously described to have a poor prognosis, with high rates of remission failure and hematologic relapse. Activating mutations in the cytokine receptor and RAS signaling pathways have been identified, including mutations in *NRAS*, *KRAS*, *FLT3*, *IL7R*, *JAK3*, *JAK1*, *SH2B3*, and *BRAF*. Inactivating mutations in genes that encode proteins required for normal differentiation, including *GATA3*, *ETV6*, *RUNX1*, *IKZF1*, and *EP300*, have also been described.

Other subtypes of T-ALL involve activating mutations in *NOTCH1*, which is associated with improved outcomes.

Patients with hereditary syndromes (including Down, Bloom, Klinefelter, and Fanconi) are predisposed to developing cytogenetic and molecular abnormalities that may lead to ALL. Additionally, patients exposed to ionizing radiation or radon may also be at an increased risk for developing the disease.

Risk Stratification

Children, Adolescents, and Young Adult Patients (up to 21 Years of Age)

Lower Risk
- Hyperdiploidy
- T(12;21) – *ETV6-RUNX1/TEL-AML1*
- Simultaneous trisomies of chromosomes 4, 10, and 17

Standard Risk
- Age 1–10 years
- WBC <50,000/μL

High Risk
- T-cell ALL
- Not meeting criteria for another category
- Extramedullary disease at diagnosis

Very High Risk (B-cell ALL only)
- Less than 1 year of age
- Philadelphia chromosome or *BCR-ABL1* fusion protein positivity
- Ph-like or *BCR-ABL1*-like gene signature
- Hypodiploidy (<44 chromosomes)
- *iAMP21*
- Failure to achieve remission with induction chemotherapy
- *MLL* rearranged disease

Adults

High Risk
- Age >35 years
- Elevated WBC at diagnosis (>30,000/μL for B-lineage and >100,000/μL T-lineage)
- Adverse cytogenetics: t(9;22), t(4;11), t(8;14), complex karyotype, low hypodiploidy, near triploidy
- Time to achieve CR >4 weeks
- Presence of minimal residual disease after induction and intensification

Treatment

Treatment for ALL usually consists of three phases: induction, consolidation, and maintenance therapy. Several different treatment regimens exist, and the choice of therapy depends on the patient's age and subtype of ALL, as well as physician preference and the patient's performance status and potential ability to tolerate certain agents (e.g., L-asparaginase). All treatment regimens incorporate prophylaxis and/or treatment for CNS disease.

Induction

Induction therapy is meant to debulk the tumor burden in the bone marrow. Most ALL induction therapy regimens are designed based on pediatric models (such as the one designed by the Berlin-Frankfurt-Münster group) and incorporate agents such as vincristine, anthracyclines, corticosteroids, and possibly cyclophosphamide and/or L-asparaginase. ALL cells are unable to synthesize the amino acid asparagine, and they require it from an exogenous source in order to proceed with protein translation. Asparaginase is utilized because it causes the rapid deamination of asparagine, depleting it from the serum and causing cell death. The agent must be used with caution, however, as it can cause LFT abnormalities, pancreatitis, hypercoagulability, and hypersensitivity reactions.

Consolidation

Consolidation therapy is given with the goal of eradicating residual disease that may persist after induction. In some regimens, the consolidation phase also includes an intensification phase, which can be considered a post-remission re-induction. Commonly used agents include high-dose methotrexate, 6-mercaptopurine, vincristine, cytarabine, corticosteroids, cyclophosphamide, and L-asparaginase.

In patients who are considered standard or high risk, allogeneic HCT should be considered as a consolidative therapy.

CNS Prophylaxis

Unlike AML, sanctuary site relapse is commonly seen in ALL. Therefore, periodic intrathecal chemotherapy, coupled with cycles of high-dose methotrexate, is administered starting during induction and continuing throughout consolidation and maintenance. Cranial irradiation is sometimes considered for adult patients who are not planning to undergo an allogeneic transplant. With CNS prophylaxis, the risk of relapse in this site decreases from 30% to 5%.

Maintenance

For patients who do not go on to a HCT, extended maintenance therapy can prevent disease relapse and has been shown to significantly improve overall survival in ALL patients. Commonly used agents included in maintenance may be omitted in patients with mature B-cell ALL, as these patients often have long-term remissions with short courses of intensive therapy, and relapses are rarely seen in this population beyond 12 months.

Tyrosine Kinase Inhibitor Therapy

BCR-ABL tyrosine kinase inhibitors are used in patients with Philadelphia chromosome-positive ALL. By inhibiting the kinase activity of the BCR-ABL protein product, these agents eliminate the constitutive downstream signaling that drives the malignancy. Both imatinib and dasatinib are reasonable options; however, the latter has increased potency in inhibiting signaling pathways, activity against various ABL kinase mutations, and greater penetration of the blood-brain barrier and therefore is commonly preferred.

In patients who do not wish to undergo intensive therapy, or are not candidates for it, TKI with corticosteroid therapy is a possible treatment option.

Anti-CD20 Therapy

Approximately 32% of patients with Philadelphia chromosome-negative precursor B-cell ALL express the CD20 antigen on the cell surface, although some estimates put this closer to 40–50%. Patients with CD20 expressing B-cell ALL may benefit with anti-CD20 monoclonal antibody therapy, e.g., rituximab. While other anti-CD20 antibodies, such as obinutzumab and ofatumumab, have been developed, they are not currently approved for use in ALL patients.

Other Targeted Therapies

The bi-specific T-cell engager blinatumomab targets CD19-positive cells and can be used in patients with relapsed/refractory Philadelphia chromosome-negative ALL. It is commonly used to induce an MRD-negative remission in patients who have achieved an MRD-positive CR. Side effects include fever, cytokine release syndrome, neurological toxicities, neutropenia, and infection.

The anti-CD22 antibody-drug conjugate inotuzumab ozogamicin is also used in patients with relapsed/refractory disease who express CD22 on their ALL cells. The most common adverse event associated with inotuzumab is hepatotoxicity, including sinusoidal obstruction syndrome (also known as veno-occlusive liver disease).

Cellular Therapy

Anti-CD19 chimeric antigen receptor T-cells (CAR T-cells) came into widespread use in 2017. Anti-CD19 CAR T-cells are developed by engineering a T-cell receptor with an extracellular domain targeted against CD19 and intracellular signaling domains of the T-cell receptor complex. This allows the T-cells to engage the CD19-positive ALL cells, which activates the T-cells and stimulates a cytotoxic response.

Tisagenlecleucel was the first anti-CD19 CAR T-cell therapy approved by the FDA for use in children and young adults with relapsed/refractory ALL in 2017. This product is an autologous CAR T-cell therapy which uses a patient's own cells. It was found to create durable, MRD-negative responses in patients with ALL, even in those who had relapsed after HSCT.

Supportive Care

Leukostasis

Patients with elevated WBC, usually >100,000/μL, may develop symptoms of leukostasis. These symptoms may include neurological symptoms such as numbness, tingling, decreased hearing, vision changes, and tinnitus, and pulmonay symptoms such as dyspnea, tachypnea, hypoxia, and cyanosis. Treatment in this situation should be individualized, but the approach may include administration of fluids (not blood products) and leukapheresis. For patients with an elevated WBC who are asymptomatic and who are not yet ready for induction therapy, hydroxyurea can be used for leukoreduction in AML, and steroids may be used in patients with ALL. APL patients with high WBC are not routinely treated with leukapheresis. See the previous discussion in this chapter regarding leukostasis in AML.

Tumor Lysis Syndrome

Tumor lysis syndrome (TLS) is defined by the Cairo-Bishop criteria as potassium >6 meq/L, phosphate >4.5 mg/dL, calcium <7 mg/dL, and uric acid >8 mg/dL. Standard prophylaxis includes hydration with diuresis and allopurinol. If TLS develops, standard treatment may include rasburicase, a recombinant urate oxidase enzyme that allows for the rapid enzymatic degradation of urate crystals. Institutional guidelines may vary on when to use rasburicase, but a mildly elevated uric acid level without other signs of ongoing TLS may not warrant the use of this costly medication. Additionally, patients should be screened for potential glucose-6-phosphate dehydrogenase deficiency prior to rasburicas administration, as methemoglobinemia may result from the production of hydrogen peroxide in the urate oxidase reaction in these patients.

Coagulopathy

Coagulopathy can occur at presentation in many leukemias, and thus all patients with acute leukemia should have a prothrombin time, activated partial thromboplastin time, fibrinogen, and platelet count (as part of the CBC) for screening. For patients with APL, platelets should be kept ≥50,000/μL, fibrinogen levels should be followed, and cryoprecipitate should be provided to maintain a level over 150 mg/dL, and PT and aPTT should be followed with treatment as appropriate.

Blood Product Support

Patients treated in the inpatient setting should receive leukocyte-depleted blood products. If possible, irradiation of blood products is also recommended to reduce infectious risk. While guidelines for inpatient management may vary across institutions, many transfuse packed RBCs for a hemoglobin <7 mg/dL to promote adequate tissue oxygenation and transfuse platelets for a count <10,000/μL to reduce the risk of spontaneous intracranial hemorrhage.

Opportunistic Infections and Neutropenic Fever

Patients who are neutropenic should be placed on viral and fungal prophylaxis given the high risk of opportunistic infections. Of note, posaconazole has been shown to significantly decrease fungal infections as compared to fluconazole or itraconazole. Isavuconazole is also gaining favor given fewer drug interactions and potentially less QT prolongation. Bacterial prophylaxis, for example, with levofloxacin, should be offered to patients who remain neutropenic in the outpatient setting. Prophylaxis should be continued until the ANC remains steadily above 1000/μL. Neutropenic fever should be treated as per local guidelines with the use of an antipseudomonal, broad-spectrum antibiotic, continued at least until the neutropenia has resolved. A rigorous infectious workup should also be pursued.

Fertility and Suppression of Menses

Patients of childbearing potential should be counseled on options for preserving fertility prior to chemotherapy initiation, if possible. Some female patients may require treatment to suppress menstruation, given the risks of hemorrhage with concurrent treatment-related thrombocytopenia. Conception should be avoided during treatment and patients should be counseled on this point.

Summary

Acute myeloid leukemia is a malignant proliferation of myeloid progenitor cells which can lead to symptoms of bone marrow failure, impaired immunity and infections, DIC, leukostasis, and CNS disease. Classification and risk stratification give information on prognosis and can change treatment recommendations. Patients are generally treated with induction therapy, most commonly using an anthracycline and cytarabine combination regimen, followed by consolidation, which may or may not include a bone marrow transplant. Acute promyelocytic leukemia is a subtype of AML which is driven by a *PML-RARα* fusion; the development of regimens incorporating all-trans retinoic acid (ATRA) has made this aggressive form of leukemia highly curable. Acute lymphoblastic leukemia is a clonal neoplastic malignancy of immature lymphocytes which more commonly presents with CNS disease. Classification and risk stratification are again important in these patients, who are treated with induction, consolidation, CNS-directed therapy, maintenance, and possibly tyrosine kinase inhibitor therapy and/or bone marrow transplantation. Supportive care measures are important to understand, as specialized treatment strategies exist for leukostasis, tumor lysis syndrome, and other complications seen with the acute leukemias.

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Classification of Lymphomas

29

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Lymphomas are a heterogeneous group of neoplasms originating from lymphocytes. They can arise from T cells, B cells, or natural killer cells, and this malignant transformation occurs at various stages of development, usually within a lymph node, the bone marrow, or the spleen, though involvement with extranodal sites can be seen as well. These various progenitor cells lead to a broad category of diseases which at times can be difficult to understand given its wide spectrum of clinical characteristics and associated syndromes [1].

Lymphomas have traditionally been separated into two major groups: Hodgkin and non-Hodgkin lymphoma. Over time, several distinct entities have been identified within Hodgkin lymphoma such as nodular lymphocyte-predominant Hodgkin lymphoma or classical Hodgkin lymphoma, which as a group contains nodular sclerosing, mixed cellularity, lymphocyte-rich, and lymphocyte-depleted Hodgkin. Non-Hodgkin lymphoma has been further subdivided into dozens of types over the years as we have refined our understanding of these neoplasms, with 84 distinct non-Hodgkin lymphoid neoplasms now recognized [1]. Classification systems have evolved significantly, initially relying on morphology and clinical behavior but incorporating immunohistochemistry, molecular data, and genetics as the technology became available (Table 29.1).

Table 29.1 Classification of lymphomas

Group	Number of major subtypes
Mature B cell neoplasms	9
Mature T and NK cell neoplasms	18
Hodgkin lymphoma	5
Posttransplant lymphoproliferative disorder	6
Histiocytic/dendritic cell neoplasms	9

Historic Approaches to Classification

The first widely used classification system for non-Hodgkin lymphoma, the Rappaport classification, pre-dates the division of lymphocytes into T cells and B cells. It was proposed initially in 1956 and later revised in 1966 [2]. It relied on three parameters: pattern of tumor, cellular type, and degree of differentiation [2]. Although various other classification systems were introduced subsequent to Rappaport, the National Cancer Institute's (NCI) Working Formulation is the one which became standard in the United States after it was proposed in 1982 [3]. This system stratified ten different histologic groups of non-Hodgkin lymphomas across three grades of clinical outcomes (low, intermediate, and high grade). It had limitations, however, with perhaps one of the more notable among them being that it did not include T cell lymphomas. In 1994, the Revised European-American Lymphoma (REAL) classification was proposed. This system was derived from consensus among a group of expert hematopathologists. It incorporated technologies that were becoming increasingly used in the clinical setting such as immunophenotyping and genetic analysis [4].

The World Health Organization (WHO) released its own WHO classification system in 2001, building upon the REAL classification. This system was updated again in 2008 and 2016. It is based on several key principles which include morphology, immunophenotyping, molecular biology, genetics, and clinical course. This combination of techniques

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informing our classification schema has allowed us to better appreciate the diversity of lymphoid neoplasms.

In the WHO system, entities are divided across five major groups: mature B cell neoplasms, mature T and NK cell neoplasms, Hodgkin lymphoma, posttransplant lymphoproliferative disorders, and histiocytic and dendritic cell neoplasm [5]. The 2016 update has added several new provisional entities which are increasingly recognized, and it has promoted some prior provisional entities into definite ones. The changes in this edition have been most notable within the mature B cell neoplasm category, reflecting the latest discoveries in chronic lymphocytic leukemia/small lymphocytic lymphoma, monoclonal B cell lymphocytosis, lymphoplasmacytic lymphoma, and many others [5].

While vital to clinical practice, this complex system can at times seem cumbersome. It can therefore often be useful to separately consider many of these non-Hodgkin entities as falling into the category of indolent or aggressive lymphomas. The clinical outcome of these two clinical groupings of lymphoma can be markedly different. Patients diagnosed with aggressive lymphomas such as Burkitt lymphoma may only live for months or even weeks in the absence of treatment, whereas those with untreated indolent lymphomas such as hairy cell leukemia may expect to live for many years. While such a distinction does not lead to the granular classification noted above, it can be extremely helpful both in discussion with the patient and in guiding our plans for management.

Diagnosis

While we have transitioned away from a purely morphologic approach to diagnosis of lymphomas, morphology remains an important element of the process. For example, while it can be impossible to reliably differentiate many of the mature low-grade small B cell neoplasms by morphology, it remains an essential part of distinguishing high-grade large B cell neoplasms such as diffuse large B cell lymphoma. When morphologic analysis alone does not reveal a clear diagnosis, we now have the benefit of immunohistochemical markers, flow cytometry, and cytogenetics which help to distinguish morphologically similar but clinically distinct entities among the B cell neoplasms. Through combination of these varied techniques, one can reach a more accurate diagnosis in the modern classification system.

Mature B Cell Neoplasms (See Table 29.2)

The mature B cell neoplasms comprise 90% of all lymphomas [6]. One of the most important diagnostic questions to answer is whether or not the disease is a low- or high-grade lymphoma. The low-grade mature B cell neoplasms are indolent diseases which include chronic lymphocytic leukemia (CLL), follicular lymphoma, lymphoplasmacytic lymphoma (LPL), and hairy cell leukemia among others. The high-grade neoplasms in this category include diseases such as diffuse large B cell lymphoma and its variants, Burkitt lymphoma, and mantle cell lymphoma.

Mantle Cell

Mantle cell lymphoma is composed of small- to medium-sized B cell lymphocytes. It is a heterogeneous entity which may demonstrate indolent or more aggressive behaviors. Because of that potential for aggressive behavior, it is important to distinguish it from morphologically similar lymphomas. As with the other B cell neoplasms, it is positive for markers such as CD19, CD20, and CD79a but lacks CD23. It can be distinguished from follicular lymphoma, as can other neoplasms, in that it generally does not express CD10. Mantle cell lymphoma also expresses CD5, a marker more commonly associated with T cells, which is a trait it shares with CLL and hairy cell leukemia. Finally, it classically is found with immunohistochemistry to overexpress cyclin D1, a regulatory protein associated with progression through the cell cycle [7]. The pathognomonic t(11;14)(q13;q32) translocation confirms the diagnosis of mantle cell lymphoma. This genetic hallmark leads to the upregulation of the cyclin D1 protein, a critical regulator of the G1 phase of the cell cycle. Specifically, the t(11;14) translocation, present in virtually all cases of MCL, juxtaposes the proto-oncogene CCND1 to the immunoglobulin heavy-chain gene. This causes cyclin D1, normally not expressed in B lymphocytes, to become constitutively overexpressed. This alteration is thought to facilitate the deregulation of the cell cycle at the G1-S phase transition.

Chronic Lymphocytic Leukemia

CLL is a mature B cell neoplasm which is indolent in its course. On morphologic review, it is characterized by an abundance of small mature lymphocytes evident in the peripheral circulation. Immunohistochemical studies will classically reveal lymphocytes positive for CD19 and dim CD20 [8]. It can be further distinguished from similar entities as it is generally positive for CD5, a trait it shares with mantle cell lymphoma and hairy cell leukemia. It can be distinguished from follicular lymphoma by its CD10 negativity and from mantle cell lymphoma as it does not overexpress cyclin D1. These morphologic and immunohistochemical characteristics usually allow for diagnosis without further studies being undertaken.

Review of a lymph node biopsy may be helpful in situations where the diagnosis is in doubt such as with atypical immunohistochemistry/morphology. The presence of pseudo-follicles in the node is classic for CLL and may help

Table 29.2 Mature B cell neoplasms

Disease	Clinical characteristics	Immunophenotype	Molecular markers	Genetic changes	Clinical pearls
MCL	Indolent to aggressive	CD5/cyclin D1+ CD23−	Cyclin D1 over expression	t(11;14)(q13;q32)	Due to its variable clinical behavior, important to distinguish this from other small mature B cell lymphomas
CLL	Indolent	CD5/CD23/dim CD20/dim sIG+ Cyclin D1−	NOTCH1, SF3B1, TP53	Trisomy 12, del 11q, del 13q14, del 17p	Often found with incidental leukocytosis on bloodwork. SLL variant presents with adenopathy without leukocytosis
FL	Indolent	CD10+ CD23+/− CD5−	BCL2 over expression	t(14;18)	High-grade follicular lymphoma can behave similarly to DLBCL. Often present with marked adenopathy due to indolent progression
ENMZL	Indolent	CD5/CD10/cyclinD1−		t(11;18), t(1;14), t(3;14), t(14;18)	
NMZL	Indolent	CD23+/− CD5/CD10/cyclinD1−		Gain 1q/2p/3p/3q/6p Loss 1q/6q Trisomy 3/12/18 Monosomy 9/13/14	
SMZL	Indolent	CD23+/− CD5/CD10/CD103/ cyclin D1−		Del or translocation of 7q32 Trisomy 3q Gain 12q Frequent complex karyotype	Often with isolated splenomegaly
LPL	Indolent	TDT+ CD10/CD20+/− CD5/sIG−	MYD88		If productive for IgM, presents as Waldenstrom's. Monitor for hyperviscosity, may need plasmapheresis
HCL	Indolent	CD20/CD25/ CD103/CD123+ sIG/CD23 +/− CD5/CD10−	BRAF V600E		Often with isolated splenomegaly. Classical hairy cells on peripheral smear
DLBCL	Aggressive	CD3−	MYC, BCL2, BCL6		Rapidly progressive, often presents with marked B symptoms but not always

Unless otherwise specified, lymphomas above are positive for pan-B cell markers (CD19, CD20, CD79a, PAX5)
MCL mantle cell lymphoma, *CLL* chronic lymphocytic leukemia, *ENMZL* extranodal marginal zone lymphoma, *NMZL* nodal marginal zone lymphoma, *SMZL* splenic marginal zone lymphoma, *FL* follicular lymphoma, *LPL* lymphoplasmacytic lymphoma, *DLBCL* diffuse large B cell lymphoma, *HCL* hairy cell lymphoma, *sIG* surface immunoglobulin

to secure the diagnosis. Bone marrow aspirate and biopsy are not required for the diagnosis, but in select situations may be quite helpful to the patient, such as in the setting of anemia or thrombocytopenia not felt to be due to hypersplenism alone but rather due to degree of marrow involvement.

In addition to morphology, cytogenetics and molecular genetics are important parts of evaluating and risk-stratifying a patient with newly recognized CLL [9]. Molecular assays can identify genetic mutations associated with poor prognosis including TP53, SF3B1, and NOTCH1, with mutation of the oncogene TP53 being the most associated with a worse prognosis of those [9]. Additionally, cytogenetic assays should be performed to evaluate for deletion in 13q (55% of cases), deletion in 11q (18%), trisomy of 12q (16%), and deletion in 17p (7%) [10]. Of these cytogenetic abnormalities, deletion in 11q (corresponding to the ATM gene) and particularly deletion in 17p (corresponding to p53) are associated with poorer prognosis, whereas deletion of 13q14 as a sole genetic is a favorable feature associated with superior survival [9, 10]. Trisomy of 12q historically had been associated with worse prognosis, though after identification of the NOTCH1 mutation, it was discovered that NOTCH1 and trisomy of 12q have a high rate in co-occurrence [11]. In the absence of NOTCH1 or other higher-risk features, trisomy of 12q is felt to be a low-risk feature [9].

Monoclonal B Cell Lymphocytosis

A distinct entity of note to be aware of is the diagnosis of monoclonal B cell lymphocytosis (MBL). This condition is characterized as the presence of monoclonal B cells in the peripheral blood with the morphologic and immunohistochemical characteristics of CLL, but not exceeding a threshold of 5×10^9/L. When using the most sensitive assays available, it can be found in over 20% of healthy adults over the age of 60 and up to 75% of those over the age of 90. It can be thought of as a state of pre-CLL, with nearly all cases of

CLL being preceded by MBL. There appears to be a difference in natural history depending on the lymphocyte count in MBL, with clonal lymphocytes $<0.5 \times 10^9$/L representing a very low risk of progression to CLL and $\geq 0.5 \times 10^9$L representing a risk of progression to CLL that would require treatment of about 1–2% per year [12].

Follicular Lymphoma

Follicular lymphoma is the most common indolent lymphoma. Most patients present with advanced disease and asymptomatic lymphadenopathy. A biopsy of an involved node should be pursued to obtain the diagnosis. On morphologic exam, the tissue is notable for prominent germinal centers which contain small cells known as centrocytes and large cells known as centroblasts. The aggressiveness of follicular lymphoma correlates with the number of centroblasts noted [13]. In 2008, the WHO classification therefore laid out a grading system for follicular lymphoma based on the number of centroblasts present in these germinal centers with grade I being defined as 0–5 centroblasts/high-power field (hpf), grade 2 being 6–15 centroblasts/hpf, and grade 3 being more than 15 centroblasts/hpf. Grade 3 is further subdivided based on the presence (3a) or absence (3b) of centrocytes [4].

Immunohistochemical analysis of follicular lymphoma is notable for expression of CD19, CD20, and CD10, but these cells will be negative for CD5. The most common finding in follicular lymphoma is a translocation between chromosomes 14 and 18 which results in the overexpression of BCL-2, an anti-apoptotic oncogene [14]. If this translocation is identified, it is useful in the diagnosis of this disease but is not pathognomonic, as normal healthy people may carry the t(14;18) just as they may harbor other abnormalities such as inv16, BCR-ABL fusion protein. While we often think of various genetic abnormalities as being inextricable from certain disease processes, assorted mutations and translocations can be seen without development of the associated cancers. This underscores cancer's nature as a complex and multistep process and also drives home that even high-risk or classic oncogenic changes can be dependent on occurring in precisely the correct cell or tissue to lead to development of cancer [15].

Marginal Zone/MALT

The marginal zone lymphomas are a group of indolent non-Hodgkin B cell lymphomas that fall into three categories: extranodal marginal zone lymphoma, nodal marginal zone lymphoma, and splenic marginal zone lymphoma. The most common type is extranodal marginal zone lymphoma also known as mucosa-associated lymphoid tissue (MALT). Patients generally present with localized disease. It most commonly originates in the stomach but can also be discovered in other parts of the gastrointestinal tract as well as in the lung, skin, salivary glands, and other sites. It can spread to other extranodal sites and can even involve the bone marrow, though this is uncommon. Morphologically, it is notable for the presence of reactive-appearing follicles containing centrocyte-like cells and occasional larger centroblasts. Another important feature that may be noted is the presence of lymphoepithelial lesions. These lesions are defined as the infiltration and distortion of epithelial structures by neoplastic lymphoid cells. While this finding is not pathognomonic, it is quite common [16].

On immunohistochemistry, MALT lymphomas express CD19, CD20, and CD79a, but do not express CD5, CD10, or cyclin D1 [17]. This same immunohistochemical pattern is also found in nodal marginal zone lymphoma. Common chromosomal abnormalities seen in MALT lymphomas include a translocation between chromosomes 11 and 18, t(11;18)(q21;q21), as well as various translocations involving chromosome 14 which contains the immunoglobulin heavy-chain variable region gene [10, 18, 19]. These translocations include t(1;14)(p22;q32), t(3;14)(p13;q32), and t(14;18)(q32;q21) [19].

Beyond the pattern of nodal rather than visceral involvement, nodal marginal zone lymphoma (NMZL) is also notable for having a higher likelihood of bone marrow involvement than MALTs. The morphology of a tissue specimen is often striking, with the neoplastic cells with their abundant pale cytoplasm expanding throughout the node but sparing germinal centers or demonstrating complete effacement of the normal nodal architecture. A variety of chromosomal abnormalities are reported in NMZL with gain of 1q, 2p, 3p, 3q, 6p, and 6q being the most frequent along with loss of 1q and 6q [20]. Trisomies of chromosomes 3, 12, and 18 are often noted, but rarer are monosomies of chromosomes 9, 13, and 14 [20].

Patients with splenic marginal zone lymphomas (SMZL) generally present with splenomegaly. While bone marrow involvement is not uncommon, the cytopenias evident in these patients can exceed what would be expected from the degree of their marrow involvement alone given the splenomegaly. In this disease, the white pulp of the spleen will be gradually replaced by neoplastic cells. Immunohistochemical analysis of these cells will appear similar to that found in nodal and extranodal marginal zone lymphomas, though with the exception that a proportion of cases will be dimly positive for CD5. The most common single chromosomal abnormality in SMZL is deletion or translocation involving 7q32 which is seen alone or in combination with other abnormalities in >40% of cases [21]. Additional abnormalities which are often seen include trisomy of 3q and gain of 12q [21]. SMZL often has a complex karyotype with three or more concurrent chromosomal abnormalities, seen in >50% of cases [21].

Lymphoplasmacytic Lymphoma/Waldenström Macroglobulinemia

Lymphoplasmacytic lymphoma is an indolent disease arising from mature neoplastic B cells. It most commonly presents with nodal, splenic, and bone marrow involvement. It is rare for there to be a visceral component to this disease. Morphologically, these small cells are often found to contain Dutcher bodies, which are cytoplasmic inclusions containing immunoglobulin that are also seen in multiple myeloma. On immunohistochemistry, these cells are found to express CD19, CD20, and BCL2 but are generally negative for CD5, CD10, and CD23. This classic immunophenotype is only seen in 58% of patients, however, with the remainder demonstrating some variable expression of CD5, CD10, and CD23 [22]. The most common mutation associated with LPL is a somatic mutation in the MYD88 gene, which is also associated with response to the oral inhibitor of Bruton's tyrosine kinase (BTK) ibrutinib [23].

When LPL is associated with overproduction of immunoglobulin, most commonly IgM, it is called Waldenström macroglobulinemia. Elevated immunoglobulin levels can lead to symptoms of hyperviscosity such as bleeding, visual changes, dizziness, headache, hearing loss, or decreased level of consciousness [24]. It can also be associated with symptoms such as peripheral neuropathy, which can present in up to 20% of Waldenström macroglobulinemia patients [25]. Direct measurement of serum viscosity can be helpful in the work-up of these patients, and measurement of serum levels of IgM, IgG, and IgA is an important part of the initial work-up.

Hairy Cell Leukemia

Hairy cell leukemia (HCL) is an indolent lymphoma arising from mature B cells. Patients classically present with fatigue, marked splenomegaly, and pancytopenia [26]. The hallmark splenomegaly of the disease is not seen as frequently as it once was due to earlier detection of disease, with more patients coming to attention due to the pancytopenia or its complications before massive splenomegaly can develop [27]. Morphologically, cells classically display delicate fronds of cytoplasm which give this disease its name [28]. Its characteristic immunophenotype is positive for CD19, bright CD20, CD22, CD25, CD103, and CD123, whereas it is negative for CD5 and CD10 and with expression of surface immunoglobulin and CD23 being variable [29]. Given that it can present with splenomegaly and pancytopenia without significant adenopathy, it is important to distinguish this diagnosis from SMZL which can have a similar presentation. SMZL will be negative for CD25, CD103, and CD123 [29]. The presence of the BRAF V600E mutation is also a characteristic finding [28].

Given the lack of prior efficacious treatments, HCL was once considered fatal despite its gradually progressive nature. Even in the subset of patients who have a very indolent course, the severe pancytopenia which today often serves as the stimulus to begin therapy would ultimately lead to their demise prior to the advent of modern therapies [26]. Early therapies, including splenectomy and chlorambucil, were of limited efficacy prior to the advent of interferon alfa which demonstrated a high initial response [27]. Interferon itself was eclipsed by the advent of nucleoside analog therapy with cladribine or pentostatin, which remain highly effective first-line treatments for HCL resulting in remissions of many years [26, 28]. Given the presence of the BRAF V600E mutation, vemurafenib has also been explored as a therapy that can be effective without the notable myelosuppression associated with the nucleoside analogs [28].

DLBCL

Diffuse large B cell lymphoma (DLBCL) is an aggressive mature B cell lymphoma which is also the most commonly diagnosed lymphoma in the Western world [30]. While it may arise de novo in a patient without predisposing conditions, it is a clinically heterogeneous entity that can develop in a variety of situations. The immunoblastic variant can arise in immunocompromised hosts such as those with HIV/AIDS and is an AIDS-defining illness [31]. It can also develop from a pre-existing indolent lymphoma such as in the Richter transformation in which classically a chronic lymphocytic leukemia gives rise to DLBCL, though DLBCL can also arise from follicular lymphoma, lymphoplasmacytic lymphoma, marginal zone lymphomas, and others. Despite the variety of situations in which it can develop, it can be cured in between half to two thirds of cases [32].

Patients typically present with a rapidly enlarging nodal mass which may be identified on examination or could be discovered incidentally or as part of work-up for another problem with an imaging study. While one would expect such patients to have dramatic symptoms such as fever, rigors, weight loss, and drenching night sweats, this is seen in only a third of patients [33]. Sixty percent of patients present with advanced disease (Stage III/IV). Forty percent of patients present with primary disease beyond the lymph nodes or bone marrow which can be in nearly any part of the body, though with a predilection for the gastrointestinal tract.

Its immunophenotype generally includes the common B cell antigens (CD19, CD20, CD22, CD79a, and PAX5) as well as CD45 and can rarely express CD5 which is associated with a worse prognosis [34, 35]. DLBCL can express CD10 which can at times make it difficult to differentiate from Burkitt lymphoma or a high-grade follicular lymphoma. Given the new WHO classification of high-grade B cell lymphomas with translocations of MYC and BCL2, in

order to differentiate this entity from DLBCL not otherwise specified, evidence for overexpression of MYC and BCL2 is obtained by FISH. Partner chromosomes are BCL2 on chromosome 18 and MYC on chromosome 8. Since this overexpression can be seen in gene amplification, it is important to pursue chromosome analysis to verify the presence of BCL2 and MYC translocations which are associated with poor outcomes. MYC translocation exclusive of other translocations is a significant adverse prognostic factor, lowering 2-year overall survival (OS) from 61–72% to 33–35% [36, 37]. BCL-2 translocations alone similarly demonstrate a negative impact on OS [38].

An important subcategory of DLBCL to be aware of is the entity formerly known as "double-hit" lymphoma. The name comes from the concurrent translocations of MYC and BCL2 and/or BCL6 which define this entity which in combination confer a particularly aggressive course and poor prognosis [39]. As of the latest revision of the WHO classification system for lymphoma, this entity is now more formally known as *high-grade B cell lymphoma with translocations involving MYC and BCL-2 and/or BCL-6* [4]. Fortunately, this aggressive entity comprises less than 10% of DLBCL diagnoses [39].

Mature T and NK Cell Neoplasms

Peripheral T cell lymphoma (PTCL) and NK/T cell neoplasms are a group of rare disorders with significant heterogeneity. Diagnosis of T cell lymphoma (TCL) is challenging in part due to the low incidence of these diseases, as they represent approximately 7% of NHL, and due to the overlap in pathologic features and absence of characteristic immunophenotypes [40, 41]. The current approach for categorizing TCL utilized in the WHO 2016 classification system is based on features such as nodal sites, extranodal sites, leukemic/disseminated status, and cutaneous sites.

To identify the prevalence and clarify epidemiologic trends of various TCL, a study was performed and collected specimens from patients diagnosed from 1990 to 2002 [41]. This allowed for review of 1320 cases selected from multiple centers in North America, Europe, and Asia to evaluate the distribution of TCL subtypes and provide further epidemiologic information. This project identified the rates of various TCL (Table 29.2): PTCL NOS (25.9%), angioimmunoblastic T cell lymphoma (18.5%), NK/T cell lymphoma (10.4%), adult T cell lymphoma (9.6%), anaplastic large cell lymphoma ALK+ (6.6%), and anaplastic large cell lymphoma ALK – (5.5%), with the remaining entities representing <5% [41]. This project was undertaken prior to the 2016 update to the WHO lymphoma classification system, and therefore there may be some shifts of these numbers in future epidemiologic evaluation.

Diagnosis

Diagnostic accuracy, as defined by consensus between pathologists, is low for T cell and NK cell neoplasms compared to other hematologic malignancies. Consistent diagnosis is improved when clinical data such as human T-lymphotropic virus-1 (HTLV-1) infection, Epstein-Barr virus (EBV) infection, age, and geographic location are taken into consideration in addition to histology, immunophenotype, and molecular genetic data [41]. Overall agreement on diagnosis by numerous pathologists in the International T-Cell Lymphoma Project was <85% when excluding the diagnoses of ALK+ ALCL, ATLL (due to HTLV1 association), and NKTCL (due to EBV association) [41].

Despite the use of clinical data in the diagnostic process, there remains variation between experts. The T cell type and immune system (innate vs adaptive) of origin is increasingly being identified for the T cell neoplasms [42]. Additionally, genetic profiling (e.g., genetic panels to differentiate among peripheral T cell lymphomas NOS) is increasing over time and is anticipated to allow for more diagnostic accuracy [43].

Nodal T Cell Lymphoma

Peripheral T Cell Lymphoma Not Otherwise Specified

Peripheral T cell lymphoma not otherwise specified (PTCL NOS) comprises a large portion of the T cell lymphoid malignancies, representing 25.9% of cases. These are mature T cell lymphomas that do not correspond to another specifically defined T cell lymphoma subset in the WHO classification. Forty-nine percent of cases include both nodal and extranodal disease, and 13% have extranodal disease alone. Despite its name, the PTCL NOS group includes several specified variants:

- Unspecified PTCL: This group represents 88.5% of PTCL NOS cases. There is ongoing research to identify genetic mutations which can predict clinical behavior and may at some point have treatment implications and further clarify the unique entities within this category.
- Lymphoepithelioid (Lennert) variant: 8.2%. This group is defined by the presence of pathologic epithelioid histiocyte abundance and is a somewhat favorable predictor.
- Follicular variant: 1.8%. This group is now recognized, given the T follicular helper (TFH) cell phenotype under a common heading with AITCL which also has TFH phenotype.
- T zone variant: 0.5%. This group demonstrates the malignant T cells predominantly in the paracortical region of the LN.

There are several important clinical factors to consider in this category of disease. HTLV infection status is very helpful in distinguishing PTCL-NOS, which is less commonly

positive, from adult T cell leukemia/lymphoma, which is more commonly positive [44]. PTCL-NOS has a higher prevalence in North America and Europe (approximately 34% compared to 22.4% in Asia) [41]. Often, PTCL-NOS presents at an advanced stage and in old age.

Prognosis is guided by pathologic factors including high proportion of transformed tumor cells (>70%) and high Ki67, both of which portend a poor prognosis [44]. EBV infection has also been consistently associated with poor prognosis. CD expression has had mixed relationship to prognosis. Finally, the International Prognostic Index is predictive of OS and failure-free survival (FFS).

Anaplastic Large Cell Lymphoma

Anaplastic large cell lymphoma (ALCL) comprises 12% of PTCL and is further subcategorized as positive or negative for anaplastic lymphoma kinase (ALK) [40]. Morphologically, the cells have small basophilic nuclei. CD30 tends to be positive in all ALCL (as well as other lymphoid malignancies).

- ALK-positive ALCL has a higher prevalence in North America compared to other regions [41]. It has a favorable prognosis compared to ALK-negative disease with a 5-year OS of 70% [45]. This disease expresses CD30, a diagnostic hallmark, and tends to occur in younger patients compared to ALK-negative disease. It has a male predisposition. ALK overexpression is a result of a chromosome translocation t(2;5) (p23;35).
- The ALK-negative group has distinct cytogenetic features. The 5-year OS is worse at 49%. However, within the ALK-negative group, various gene rearrangements can help with more accurate prognostication. These genetic findings include the DUSP22 and TP63 rearrangements, which are mutually exclusive, and present in 30% and 8% of ALK-negative ALCLs; both are absent in ALK-positive ALCLs [46]. The DUSP22 gene rearrangement is associated with a favorable 5-year survival of 90%. Conversely, TP63 gene rearrangement is associated with an unfavorable prognosis, with a 5-year survival of 17%.

Interestingly, ALCL cells demonstrate loss of T cell antigens, often negative for CD3 and CD5 but positive for CD2 and CD5. Molecular testing can be done to identify T cell receptor clonality. ALK positivity has therapeutic implications as it allows for ALK-targeted therapy. CD30 testing is important in this group for more than diagnostic purposes alone because of the availability of brentuximab vedotin (BV), an antibody-drug conjugate targeted against CD30 which is approved for the treatment of systemic ALCL as second-line therapy [47]. While there is disparity in outcomes based on ALK status as noted, this disease as a whole has a better prognosis compared to other T cell neoplasms.

Breast Implant Anaplastic Large Cell Lymphoma (BIA-ALCL)

Various types of breast prostheses have been associated with BIA-ALCL, but there is a greater association with silicone-containing prostheses, and it is seen far more frequently than not in textured prosthesis. Most cases have an indolent course. They generally have an associated seroma. On analysis of the cells, they are generally T cell in origin and are found to be positive for CD30 but negative for CD15 and ALK. The pathogenesis is thought to be related to silicone stimulation of macrophages. This should be clarified as breast implant associated and differentiated from nodal ALCL and primary breast or cutaneous ALCL. The BIA-ALCL tends to be isolated in location rather than disseminated, and adjacent to one implant (not bilateral). Diagnosis can be made on cytology from seroma fluid [42, 48, 49].

Angioimmunoblastic T Cell Lymphoma

Angioimmunoblastic T cell lymphoma (AITL) comprises 18.5% of PTCL and 1–2% of all NHL [40]. Its histology is notable for effaced nodal structures with minimal retained normal follicle. The neoplastic cells often extend beyond the lymph node capsule with the subcapsular sinus preserved but dilated [51]. Cells typically stain positive for CD3, CD4, and IL-21. AITL has a higher prevalence in Europe (28.7%) and Asia (17.9%) compared to North America (16%) [50]. T follicular helper cells are the cells of origin. As a result, they often promote expansion of B cells, supported by the presence of CD10, CXCL13, ICOS, SAP, BCL6, PD1, or CCR5. Clinical features of hypergammaglobulinemia, generalized lymphadenopathy, rash, and autoimmune phenomena support a diagnosis of angioimmunoblastic T cell lymphoma [44, 50]. Reed-Sternberg cells can be present in this setting, so careful differentiation from Hodgkin lymphoma is required.

Generally, the prognosis for AITL is poor with a 5-year OS of 35%. In determining the risk posed by any given patient's disease, it is worth noting that the International Prognostic Index (IPI), used for prognosis in numerous other lymphomas, is not a strong prognostic model for AITL. The Prognostic Index for AITL (PIAI) has been created and is more accurate in predicting risk of disease and is calculated with factors including age, performance status, extranodal site, B symptoms, and platelet count [50]. Ki67 >30% is an independent risk factor when controlling for the PIAI factors [50]. Pathology can show positivity for EBV and/or HHV6B, though whether this is causative or is merely a correlation is not known [50].

Extranodal T Cell Lymphoma

The extranodal diseases are a minority of TCL and are defined primarily by their anatomic site.

Hepatosplenic gamma-delta TCL is seen in children and young adults with infiltration of the hepatosplenic tissue by immature nonactivated T cells which results in hepatosplenomegaly. Cells typically have 7q isochromosome and are positive for CD2, CD3, and CD7 but negative for CD4 and CD5, while CD8 expression is variable [40]. There is now an established association with immunosuppression such as from organ transplant and autoimmune conditions.

Enteropathy-associated TCL (EATL) accounts for 4.7% of cases of PTCL, and prevalence is higher in areas where celiac disease is of higher incidence (North America, Europe) [40]. EATL (previously identified as Type I EATL) are cases associated with celiac disease. These tend to be pleomorphic and positive for CD3 and CD7 but negative for CD56.

Monomorphic epitheliotropic intestinal T cell lymphoma (previously Type II EATL) is a monomorphic process which is positive for CD56, CD8, and MATK and not associated with celiac disease. This is more common in Asia. Often there is multifocal bowel disease and an aggressive clinical course.

NK subtype mucosal PTCL. This is more common in Asian countries compared to 4–5% of cases in Europe and North America and is associated with EBV in all cases [41]. It manifests as nasal or midline facial tumors. It can be associated with a hemophagocytic syndrome. There can be other NK extranodal sites including soft tissue or respiratory tract.

Panniculitis subcutaneous T cell lymphoma alpha-beta subtype demonstrates on pathology a T cell infiltration around the adipose cells, positivity for CD3 and CD8, negativity for CD4, and subcutaneous nodularity. Work-up should evaluate for typical panniculitis and other systemic disease such as lupus.

Leukemic T Cell Lymphoma

Adult T cell leukemia/lymphoma (ATLL) accounts for 9.6% of PTCL [40]. This disease is associated with the HTLV-1 retrovirus which integrates into T cells [40]. There is a higher prevalence in the Asian geographic region with 22.4% of all PTCL being in Asia compared to 1–2% in Europe and North America [41]. HTLV-positive status is more suggestive of ATLL rather than PTCL-NOS which can appear similar histologically [41].Patients often have had immunosuppression and can present with splenomegaly, hypercalcemia, skin lesions, and lymphadenopathy, generally with only a small disease burden detected in the peripheral blood [42].

T cell prolymphocytic leukemia (T-PLL) is a mature T cell neoplasm which previously had an overlap diagnosis with T-CLL. Clinically, the patient often presents with hepatomegaly, lymphadenopathy, and splenomegaly and more commonly is of middle age. Diagnosis can be confirmed with a chromosome 14 abnormality, often inversion [51].

T cell large granular lymphocytic leukemia (T-LGL) is a clonal T cell chronic disorder. This entity is confirmed by the presence of STAT3 and/or STAT5B mutation.

Aggressive NK and NK-like T cell leukemia/lymphoma is a disorder most commonly diagnosed in Asia. This can present with B symptoms, organomegaly, and hemophagocytosis.

Cutaneous T Cell Lymphomas

The cutaneous T cell lymphoma category has multiple categories within it defined by the specific phenotypes and origins of the neoplastic cells.

Primary cutaneous anaplastic large cell lymphoma and *lymphomatoid papulosis* are both CD30-positive cutaneous T cell lymphomas. Lymphomatoid papulosis (LP) is a disorder which typically can fluctuate in severity over numerous years with spontaneous resolution of skin lesions in some cases [52]. Primary cutaneous anaplastic large cell lymphoma is characterized more often by a few grouped lesions or a single lesion which can rapidly grow or evolve into larger tumors or thick plaques. Especially in LP, observation is warranted for small areas of skin involvement because this can be isolated in a small area and patients can have spontaneous resolution and a very indolent course.

Primary cutaneous small/medium CD4-positive T cell lymphoproliferative disorder is derived from TFH cells and is typically indolent and managed with local control.

Primary cutaneous acral CD8-positive TCL (provisional). Typically, this disease is isolated to a single site, commonly the ear. The disease has an indolent course.

Cutaneous gamma-delta T cell lymphoma has poor outcomes compared to the alpha-beta type of panniculitis. These are of cytotoxic T cell origin and the disease is not associated with EBV.

Sezary Syndrome/Mycosis Fungoides

Historically these diseases were described as two entities on a disease spectrum. Both have CD4 T cell origin. Sezary syndrome has a leukemic phase and exfoliative erythroderma. Both are CD2+, CD3+, CD5 +, CD4+, and CD8– [42]. Mycosis fungoides has a spectrum of severity and tends to be more indolent than Sezary syndrome. Mycosis fungoides can evolve over time from flat and erythematous skin patches to larger plaques with ulceration [51].

PTCL Treatment and Prognosis (Table 29.3)

For the most part, a diagnosis of PTCL portends a poor prognosis in comparison with B cell lymphomas. If localized disease is present, prognosis is clearly superior, and consideration should be given for use of radiation therapy. In systemic disease, treatment options include high-dose chemotherapy and

Table 29.3 PTCL/NK lymphoma key features

Disease (% of PTCL, as determined by the JCO International T-Cell Lymphoma Project)	Clinical characteristics	Geographic sites of higher prevalence	Immunophenotype	Molecular markers of therapeutic importance/ associated genetic changes Associated genetic changes	OS/prognosis
PTCL-NOS (25.9%)	M:F 2:1, advanced stage at dx (70%), middle age	Most common subgroup in North America, Europe			5-year OS 36%
ALCL ALK+ (6.6%)	Younger patients (10–20 years old) (compared to ALK disease)			ALK + CD30 positive	OS 79% with anthracycline-based chemotherapy
ALCL ALK – (5.5%)	Older adults (peak in six decades of life)			CD30 positive DUSP22 rearrangement	5-year OS 46%
Angioimmunoblastic (18.5%)	Older adults, diffuse LAD, hypergammaglobulinemia, skin rash, fevers, autoantibodies including autoimmune cytopenias	More common subgroup in Europe and Asia	TFH phenotype		5-year OS 33%
NK/T cell lymphoma		Asia		EBV	
Adult T cell leukemia/ lymphoma (9.6%)	Splenomegaly, hypercalcemia, skin lesions, lymphadenopathy	Asia (Japan) and the Caribbean	T reg cells express CD25, FOXP3	CCR4 HTLV-1 favors dx of ATCL rather than PTCL NOS	

possibly stem cell transplantation. Evaluation for CD30 should be done in select diseases (discussed above) given the availability of brentuximab as a therapeutic option for relapsed/refractory disease [47]. While CD30 abundance is not predictive of response, the presence of CD30 supports the use of brentuximab vedotin. ALK testing is indicated both to assess prognosis and for treatment considerations given the availability of ALK-inhibiting agents such as crizotinib. TFH identification through genetic testing may soon have therapeutic implications. The provisional classification *nodal peripheral T cell lymphoma with TFH phenotype* encompasses follicular PTCL, angioimmunoblastic TCL, and other nodal PTCL with TFH phenotype. Currently this designation does not have therapeutic implications. There will continue to be additional testing which is indicated as new targeted therapeutic options become available.

Hodgkin Lymphoma

First described by Thomas Hodgkin and Samuel Wilks in the early nineteenth century, Hodgkin lymphoma (HL) has long been identified as being a distinct entity within hematologic malignancies [53]. HL is the most common lymphoid malignancy in patients younger than 40 years of age. The two distinct categories within HL are classical Hodgkin lymphoma (CHL) and nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL). The diagnosis of HL is contingent on the presence of Reed-Sternberg (RS) cells/lymphocyte-predominant (LP) cells, which are the minority of cells present in the pathologic specimens, typically less than 1% of the total tumor specimen [54]. The specific subtype diagnosis is based on the surrounding tissue architecture and inflammatory cell composition and is clarified by immunohistochemistry (IHC) testing. RS/LP cells are characteristic multinucleated giant neoplastic cells derived from germinal center (GC) B cells in the overwhelming majority of cases (Images 1, 2, 3) [55]. Although the neoplastic cells are the minority of the parenchyma, it is the interactions with the surrounding inflammatory microenvironment that are now known to be essential in the HL pathophysiology. It is important to note that RS cells are not pathognomonic for HL and can be found in other hematologic malignancies as well as in some solid tumors and infectious processes.

Classical Hodgkin Lymphoma (CHL)

CHL constitutes the majority of Hodgkin lymphoma, representing 95% of all diagnoses. There is a bimodal incidence curve with one peak in young adults 15–34 years of age and a second peak after the age of 59 [53]. The prevalence of HL subtype varies by geographic region. In industrialized nations, nodular sclerosing HL is the most common subtype and comprises >70% of all CHL [56]. Typically, in industrialized nations patients present with B symptoms as well as painless localized lymph nodes, usually in the cervical region. In all CHL, the characteristic RS cells are CD30 posi-

tive [55]. CD15-positive staining is present in the majority of cases as well but is less specific as it can be seen in other lymphoid malignancies. In all subtypes of CHL, RS cells are surrounded directly by CD4+ T cells. These cells do not have a GC phenotype, despite being of GC origin.

- Nodular sclerosing CHL (NSCHL) is characterized by a nodular pattern with sclerosis and the presence of lacunar cells (Fig. 29.1 Image 4) [55]. The nodular component can vary with degree of lymphocyte predominance [55]. The sclerosis refers to the dense fibrotic collagen bands which subdivide the lymph node into large nodules. Variant RS cells known as lacunar cells are prevalent and are characterized by their retracted cytoplasmic membrane, resulting in a wide rim of cytoplasm. Because of the morphologic features, differential diagnosis for NSCHL includes anaplastic large cell lymphoma (ALCL); differentiation is based on IHC and molecular features. NSCHL most commonly presents with mediastinal disease and, unlike other subtypes of CHL, is most commonly seen in women [56].
- Mixed cellularity HL shows a pattern on histology without significant fibrosis and mixed cell types including lymphocytes, plasma cells, eosinophils, neutrophils, and histiocytes. Often the nodal architecture is obliterated. Classic RS cells are present. This category is typically 10–25% of cases, second in prevalence to NSCHL [56]. HIV infection can be an associated finding.
- Lymphocyte-rich CHL (LRCHL) is characterized by an abundance of cellularity in a nodular background, including histiocytes, with no eosinophils or neutrophils, with positive IHC staining for CD30 and CD15, and less frequently positive for CD20 and CD79. This accounts for 6% of cases of CHL [55]. EBV positivity is found in 50% of LRCHL (contrasting negative EBV found in NLPHL).
- Lymphocyte-depleted CHL, a minority of cases (1–5%), has sparse lymphocyte infiltration surrounding abundant RS cells. Clinically, this variant has a worse prognosis and often presents in a more advanced stage and at an older age. Two variants exist of LDCHL: a fibrotic variant with prominent fiber formation, though lacking organized collagen bands, and a reticular/sarcomatous variant which demonstrates a high number of RS cells [55].

Prognostic Factors

In Hodgkin lymphoma, the first-line therapy rate of cure is high. However, approximately 25% of patients experience refractory disease or relapse. Clinical features such as age, stage at diagnosis, and bulky disease are relevant to prognosis. For advanced stage at diagnosis, the International Prognostic Store (IPS), a model introduced in 1998 based on the clinical factors of age, sex, and blood counts, is used to predict outcomes. Pathologic factors are also known to correlate with response and have the potential to guide clinical management approaches, especially as novel therapy approaches evolve. Numerous tests related to the RS cells, the microenvironment (chemokines/cytokines and expression of CD68+ macrophages), genetics (CSF1R expression, 9p24.1 amplification), and EBV viral testing have been shown to have prognostic implications [57, 58]. A recent retrospective evaluation of patients with classical HL showed that higher 9p24.1 amplification (which increases PD-1 expression and immune evasion) was more common in advanced stage disease at diagnosis [59]. EBV most commonly is found to be positive in LRHL, LDHL, and MCCHL. The exact prognostic implications of EBV in all HL are still being studied [58]. Over time, as these tests are verified in regard to risk stratification and as treatment implications come to light targeting cell interactions and the microenvironment, some of these may become standard of care for frontline testing [54, 57, 58]. Currently therapies targeting the microenvironment are more common in the relapsed, clinical trial setting.

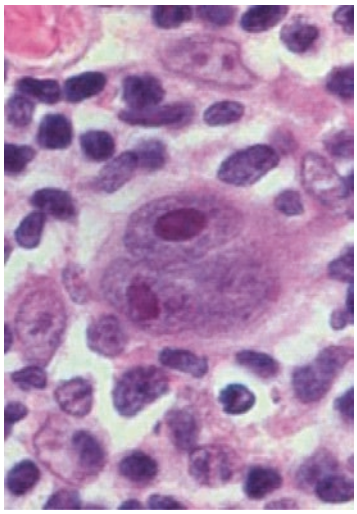
Nodular Lymphocyte-Predominant HL

Historically grouped within the Hodgkin lymphoma classification because of what was previously thought to be RS cells, this lymphoma characteristically is found to have cells now referred to as lymphocyte-predominant (LP) cells. Clinically the nodular lymphocyte-predominant subtype more resembles an indolent T cell-rich B cell lymphoma. Hence, it is crucial to differentiate this entity from CHL and follicular lymphoma because relatively to those entities, it has significantly different prognosis, age of onset, molecular testing, and treatment, among other clinical features [60]. Nodular lymphocyte-predominant HL (NLPHL) comprises 5% of HL cases. Age of onset is typically from 30 to 50 years of age and it is more common in males than females.

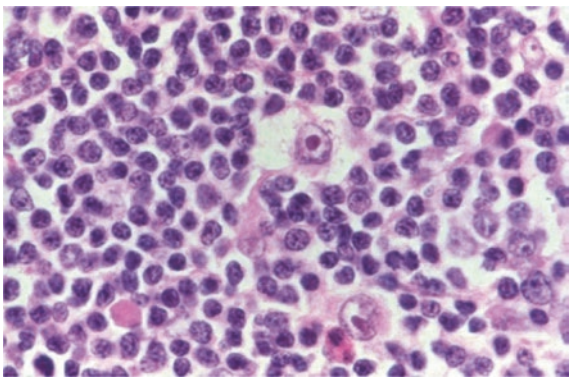
The pathology of NLPHL has unique features which differentiate it from classical Hodgkin lymphoma. In NLPHL, the Reed-Sternberg-like cells are referred to as lymphocyte-predominant (LP) cells, or "popcorn cells" (see Fig. 29.1). These cells have a unique multi-lobulated nucleus with small nucleoli and are of B cell origin [55, 60]. Evaluation of lymph nodes most often shows a nodular, interlocking background with the presence of LP cells. Additionally, the microenvironment of NLPHL is different than that of CHL. The T cells surrounding the LP cells are T helper cells (expressing CD57, PD1, and BCL6), and the surrounding follicular environment appears GC-like [61].

IHC is used to confirm the presence of LP cells, thereby diagnosing NLPHL rather than CHL or T cell histiocyte-rich LBCL (THRLBCL) [55]. IHC shows CD45+, CD20+, CD79a+, J chain+, EMA+, and PU.1+, Oct2 and BOB1,

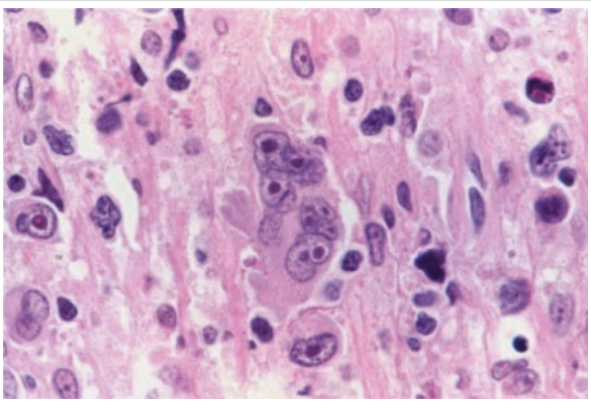
Classic RS cell
(Image 1)



RS cell with surrounding inflammatory
cells (Image 2)



RS cells surrounded by lower degree
of inflammatory cells (Image 3)



Nodular Sclerosing HL
(Image 4)
Nodules and sclerosis surrounding

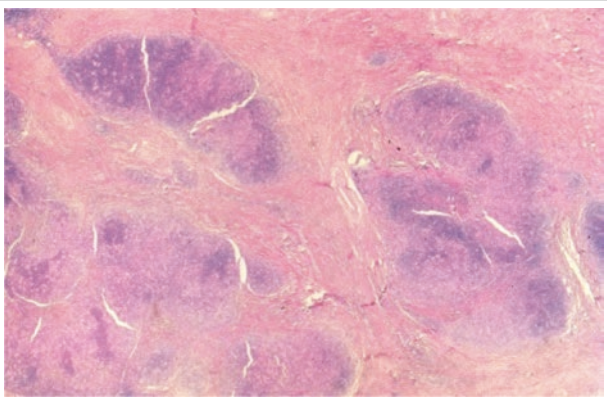


Fig. 29.1 Histopathology of Hodgkin's disease. (All images are obtained from Strong Memorial Hospital, Pathology Department)

variable CD15, and weak and/or rare CD30 [55]. CD3+ T cells surround the LP cells [60]. Additionally, EBV tends to be consistently negative in the NLPHL as opposed to CHL.

Aside from pathologic differences, clinical features can also help to support the diagnosis of LPNHL over CHL. Age of onset for LPNHL is middle age, and the disease is male predominant, as opposed to CHL which has a bimodal age of onset. Typically, this disease is unifocal rather than multifocal. Given the indolence of this disease and high rate of late recurrence, accurate diagnosis up front is imperative to differentiate from CHL. If T cells are prominent, NLPHL can also be confused with THRLBCL which is clinically a very aggressive disease. If a diagnosis of NLPHL is verified based on detailed immunophenotyping as well as careful inspection of the architecture of the (ideally) excisional biopsy but does resemble THRLBCL, this is then referred to as NLPHL, THRLBCL-like, rather than transformed lymphoma [60].

Posttransplant Lymphoproliferative Disorder

Posttransplant lymphoproliferative disorder (PTLD) describes a range of lymphoproliferative conditions that occur in the setting of both allogeneic hematopoietic stem cell transplant and solid organ transplant [62]. It occurs in less than 2% of transplanted patients and can manifest as anything from a reactive polyclonal process to overt high-grade lymphoma [63].

Epstein-Barr virus (EBV) is a major contributor to PTLD, either being present in latent infected cells in a transplant recipient or being transmitted to an EBV-negative recipient by an EBV-positive donor [64]. While at one point the clear majority of PTLD cases were felt to be EBV related, an increasing proportion of EBV-negative PTLD cases has been described over time reaching about 30% of cases [4].

Previously there were four categories of PTLD in the 2008 WHO classification system: early-type, monomorphic, polymorphic, and classical Hodgkin lymphoma. The latest iteration of the WHO's system has removed early-type PTLD as a formal category and has replaced it with plasmacytic hyperplasia PTLD, infectious mononucleosis PTLD, and florid follicular hyperplasia PTLD, which had all previously been described as subdivisions of early-type PTLD.

Monomorphic PTLD

Perhaps the simplest of the PTLD subtypes to diagnose, monomorphic PTLDs are lymphomas that develop in the posttransplant setting which fulfill the diagnostic criteria of other lymphomas such as diffuse large B cell lymphoma, Burkitt lymphoma, or T cell lymphomas [4].

Polymorphic PTLD

In polymorphic PTLD, an infiltrate is appreciated which consists of a variety of cells such as plasma cells, B lymphocytes in various stages of maturation, T lymphocytes, and histiocytes. As a result of this heterogeneous mixture of cells, no single classic immunophenotype is demonstrated [63]. Molecular analysis of IGH or episomal EBV genome may demonstrate a clonal pattern [65].

Classical Hodgkin PTLD

Classical Hodgkin PTLD is a rare but described entity in the literature. As with classical Hodgkin lymphoma, classical Hodgkin PTLD will have Reed-Sternberg (RS) cells positive for both CD15 and CD30. However, other forms of PTLD can have RS cells as well. Unlike the typical RS cells seen in classical Hodgkin PTLD, these other cases of PTLD will have RS cells that are positive for CD30 but generally lack expression of CD15 as well as CD45 [66].

Plasmacytic Hyperplasia PTLD

Plasmacytic hyperplasia PTLD previously was included as a morphologic subtype of the prior early-type PTLD category. It is characterized by numerous plasma cells with rare immunoblasts [63, 65]. It can be difficult to distinguish from infectious mononucleosis PTLD as some cases can have morphologic features of both conditions, and in both cases the immunophenotype is polytypic and clonal cytogenetic changes are not common.

Infectious Mononucleosis PTLD

Infectious mononucleosis PTLD is characterized by marked paracortical expansion of an involved node by a mixed population of both plasma cells and T cells with prominent immunoblasts [63, 65]. As noted above, it can be difficult at times to distinguish this from plasmacytic hyperplasia PTLD.

Florid Follicular Hyperplasia PTLD

Florid follicular hyperplasia PTLD is now included in the WHO classification system for lymphoma given increasing evidence that it represents a form of PTLD [66, 67]. Patients typically present with adenopathy which on biopsy was found to have enlarged germinal centers with prominent tingible body macrophages. As with infectious mononucleosis PTLD and plasmacytic hyperplasia PTLD, the immunophenotype demonstrates a polytypic profile given the variable mixture of cells that are present. While it has been described as an early or precursor lesion of PTLD, pre-existing florid follicular hyperplasia PTLD does not generally progress to another form of PTLD.

Histiocytic and Dendritic Cell Neoplasms

Histiocytic and dendritic cell neoplasms are a heterogeneous and relatively rare group of diseases that comprise less than 1% of lymphocyte neoplasms. Given the rarity of these neoplasms, it can be challenging to make one of these diagnoses that requires the aid of a skilled hematopathologists. The various entities within this class can often be easily confused for one another or other malignancies such as soft tissue sarcomas, skin cancers such as melanoma, or metastatic diseases of unknown primary.

Langerhans Cell Histiocytosis

Langerhans cell histiocytosis, also referred to as histiocytosis X in some literature, is the most common neoplasm of its category and carries with it a broad range of clinical manifestations ranging from minor, to potentially fatal depending on severity of the disease and the areas of involvement. While it can occur at any age, it is most common in children [68]. It can be either a unifocal or a multifocal to disseminated process. It is more common for children and adults to have a unifocal presentation, whereas infants are more likely to have multiorgan involvement. It is unlikely but well-described for a unifocal presentation to progress to a multifocal one, with this happening about 10% of the time.

Biopsy will reveal mononucleated histiocytes with classically coffee- or kidney-bean-shaped nuclei. Immunohistochemistry can help to distinguish this condition from most other histiocytic neoplasms as LCH cells will be positive for S-100, CD1a, and CD207 [69].

Langerhans Cell Sarcoma

Langerhans cell sarcomas are extremely rare malignancies occurring most frequently in adults of middle age [4]. They have been reported as a de novo process as well as developing from Langerhans cell histiocytosis [70]. They have also been reported as arising in patients with lymphomas with some clonal relation between the two neoplasms [71, 72]. Prognosis is generally poor with limited response to systemic therapies. As with LCH, these neoplasms will be positive for CD1a and S-100. Despite its similar immunohistochemical profile, it can be distinguished by what is described as a frankly malignant set of cytologic features [73].

Histiocytic Sarcoma

Prior to the rise of modern immunohistochemical techniques and genetic testing, histiocytic sarcoma was a more common diagnosis to render. In truth a rare neoplasm derived from monocytic/macrophage cells, it can be confused with a variety of other diseases based on morphology alone. It can arise in either the lymph nodes or extranodal sites such as the skin or gastrointestinal tract [74]. Some cases present with multifocal/systemic disease, which appears to be associated with a more aggressive course and worse prognosis. Unfortunately, most patients with this diagnosis do end up dying of their disease [73, 74]. In terms of its immunophenotype, it stains positive for leukocyte common antigens, CD68, lysozyme, and generally CD4. Half of cases are positive for S-100 [73].

Given the rarity of this diagnosis, literature pertaining to it is sparse, especially given the challenge of correctly identifying cases of this disease prior to modern techniques which renders older literature suspect [75]. As a result, there is no single approved therapy, though depending on the nature of the presentation surgery, radiotherapy, and systemic chemotherapy have all been employed.

Interdigitating Dendritic Cell Sarcomas

Derived from mesenchymal dendritic cells, interdigitating dendritic cell sarcoma is a rare disease. In adults, it is most commonly a disease of the lymph nodes, though in pediatric cases it is more prone to extranodal tumors [76]. Its immunophenotype is characterized by intense expression of S100 while being negative for CD1a and CD207 [77]. Often described as a very aggressive and fatal disease, there may be some cases with a more benign prognosis based on case reports [73].

Indeterminate Dendritic Cell Tumor/Sarcoma

Indeterminate dendritic cell neoplasm is a rare diagnosis that generally presents as cutaneous disease. Over 90% of described cases are cutaneous, most often manifesting in a nodular or popular form. While generally a disease of adults, there has also been a congenital disease described [78]. Non-cutaneous forms have been described in the literature, in one case reported as a systemic disease in a newborn and in another as primary splenic disease in an elderly woman [79, 80]. Approximately a fifth of cases are reported as being discovered in association with another hematopoietic neoplasm [81]. On biopsy, a proliferation of histiocytoid cells will be noted which are positive for CD1a, sometimes positive for S-100, and negative for langerin.

Follicular Dendritic Cell Sarcoma

Follicular dendritic cell sarcoma (FDCS) is a low-grade neoplasm derived from mesenchymal dendritic cells [68]. It tends to present as an indolently growing mass which is often painless [81, 82]. It can be found in individuals of any age but most commonly is seen in individuals of middle age and can present as either nodal or extranodal disease [83]. FDCS generally presents as localized disease but does have the potential to metastasize to the lungs, liver, or lymph nodes [84]. These neoplasms are generally positive for CD21, CD23, and CD35.

Fibroblastic Reticular Cell Tumor

Fibroblastic reticular cell tumors (FRCT) are exceedingly rare neoplasms with around 20 cases being reported in the literature to date [83]. Given the sparse information available about cases of this neoplasm, its presentation and clinical behavior are challenging to predict. In the available reports, it generally has followed a course comparable to a low-grade sarcoma, though a more aggressive course is predicted by intra-abdominal disease [84]. Though described most frequently as a local disease, metastases to the liver have been described in a few cases, and more widespread disease to the liver, bone, and lymph nodes in one patient [84]. On immunohistochemistry, FRCT cells are noted to be positive for vimentin, smooth muscle actin, and desmin while being negative for CD21, CD35, and S-100.

Disseminated Juvenile Xanthogranuloma

Disseminated juvenile xanthogranuloma (DJXG) is a neoplasm characterized by development of erythematous or yellow cutaneous nodules starting in early childhood. Rarely, there has been reported development of these nodules in the subcutaneous tissues or skeletal muscle. There has also been involvement of the viscera or bone reported in rarer cases [85]. The biopsy of classic DJXG reveals foamy lipid-laden histiocytes and occasional Touton giant cells, though there are other histologic subtypes that are reported. Diagnosis is aided by immunohistochemistry. As may be expected, these cells stain for markers classic in macrophages such as CD68, anti-FXIIIa, and vimentin. They can be positive for anti-CD4. They can be distinguished from Langerhans cell histiocytosis by their S-100 and CD1a negativity.

Clinical course for this neoplasm can range from isolated and self-limited lesions which can be safely observed to more extensive or visceral involvement meriting systemic therapy.

Erdheim-Chester Disease

Erdheim-Chester disease generally affects adults aged 55–60, only rarely being diagnosed in children, and is more common in males at a rate of 3:1 [86]. The majority of patients, over 90%, present with osseous involvement, and 50% of patients report associated skeletal pain [87]. Imaging can be helpful and is often pursued given bone pains endorsed by patients. Bone scan or positron emission tomography (PET) can be specific for characteristic diaphyseal and metaphyseal osteosclerosis, with the latter being less specific [88]. Plain film X-rays may often be obtained prior to formal diagnosis, revealing sclerotic areas at the diaphysis and metaphysis of bones. Other common manifestations are CNS and cardiovascular involvement, fibrosis of the retroperitoneum, and exophthalmos.

While biopsy is required for diagnosis of ECD, it is not diagnostic in itself but merely serves as another important factor alongside clinical evaluation and imaging studies [89]. The classic finding on biopsy is foamy, lipid-laden mononucleated histiocytes generally with concurrent fibrosis. Immunohistochemistry can help to distinguish this from other conditions. These histiocytes are positive for CD68 and CD163. Unlike Langerhans cell histiocytosis, ECD cells are negative for CD1a, though diagnosis can be clouded by concurrent LCH lesions within an ECD biopsy.

Summary

The biological diversity of lymphoma has necessitated increasingly complex classification methods, culminating most recently in the 2016 update to the WHO's classification system. That very complexity that allows us to more precisely characterize a given patient's disease can, however, make the prospect of learning about this topic quite daunting. This difficulty is compounded by the rarity of many of the conditions described. By approaching the question of classification in a systematic way starting with the five broad groups utilized by the WHO, and further informing the diagnosis through clinical correlation and potentially by drawing on modern methods of immunohistochemistry and cytogenetics, one can be more confident in the diagnosis. It is important to recognize, however, that this classification system is dynamic, changing over time to reflect our growing understanding of lymphoma biology, as evidenced by the recent inclusion of several provisional entities. As time goes on, we will undoubtedly see even further nuance added to this broad and fascinating group of cancers.

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Clinical Evaluation and Management of Hodgkin Lymphoma

30

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Introduction

Hodgkin lymphoma (HL), formerly called Hodgkin's disease, is a malignant disorder arising from B-cell and rarely T-cell lymphocytes present in the lymphoid system. It was first described by Sir Thomas Hodgkin in 1832. The characteristic hallmark multinucleated "Reed-Sternberg cells" (RS cells) that define the disease were named after Carl Sternberg and Dorothy Reed who separately described these cells in 1898 and 1902, respectively. HL commonly affects two different age groups; the first peak involves adolescents and young adults, while the later peak affects adults older than 50 years of age. Patients typically present with painless lymph node enlargement with or without other constitutional symptoms. Therapy for Hodgkin lymphoma has evolved over the years. Currently, the mainstay of treatment is combined chemotherapy and radiotherapy. Although survival is excellent with this modality, long-term toxic treatment complications are common.

Epidemiology and Pathology of Hodgkin Lymphoma

A. *Epidemiology*
   1. HL is one of the most common malignancies in adolescents and young adults. In the United States, it is estimated that there were about 8500 cases of HL in 2016 and approximately 1100 deaths in the same year.
   2. HL has a bimodal age distribution; it peaks at 20–30 years of age and again after 50 years. Based on Surveillance, Epidemiology and End Results Program (SEER) data, in 2016 more than 30% of cases of HL occurred in people aged 20–34 years.
   3. There is an association with socioeconomic status, with a higher incidence reported in the industrialized nations. In the developing countries, younger children are more commonly affected. HL has been associated with Epstein-Barr virus (EBV) infection in a subset of cases, but the precise pathophysiologic mechanism has not been well elucidated.
   4. HL has an excellent prognosis. Overall survival in the past 10 years has exceeded to 90% with current treatment approaches.

B. *Pathology*. HL is divided into two main classes: classical HL which accounts for about 95% of cases and nodular lymphocyte-predominant HL (NLPHL).
   1. *Classical Hodgkin Lymphoma*. This is characterized by the presence of RS cells (Fig. 30.1), surrounded by a rich reactive mixed cellular infiltrate of histiocytes, lymphocytes, plasma cells, and polymorphonuclear cells including neutrophils and eosinophils. Less than 5% of the tumor is comprised of the neoplastic RS cells, which on immunohistochemistry staining are uniformly positive for CD30 and typically CD15 (Fig. 30.2). CD30 is a cell membrane protein which is a member of the tumor necrosis factor receptor family and is expressed on small subsets of activated B- and T-cells. It is very highly expressed in RS cells and also subsets of non-Hodgkin lymphoma, most notably anaplastic large cell lymphoma. RS cells are derived from germinal center B-cells that are unable to express surface immunoglobulin due to a deficiency in the immunoglobulin promoter transcriptional activators – octamer-binding transcription factor 2 (Oct-2) and B-cell Oct-binding protein 1 (Bob-1). The RS cells also harbor alterations in the short arm of chromosome 9 (chromosome 9p24.1), the locus for programmed death-1 ligands (PD-1 ligands). PD-1 ligands are examples of immune checkpoint proteins

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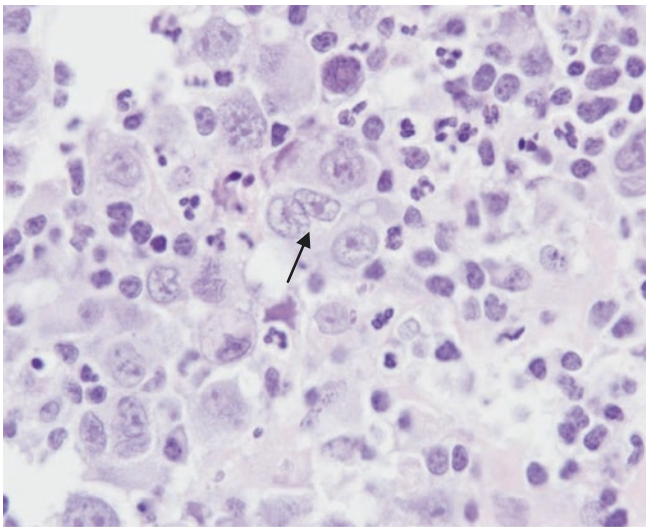

Fig. 30.1 H&E stain of classical Hodgkin lymphoma. Reed-Sternberg cell (arrow) "owl's eye appearance" is surrounded by a mixed inflammatory infiltrate. (Image courtesy of Dr. S. Chen, Department of Pathology, Boston Children's Hospital, Boston MA)

and allow tumor cells to evade the immune system. Classical HL is further subdivided into four histology variants. See Table 30.1 for the epidemiological and clinical differences between these subtypes.

(a) *Nodular Sclerosis*. It is the most common variant of classical HL, accounting for about 70% of cases of HL. Morphologically it is defined by nodular aggregates surrounded by dense fibrous collagen bands (Fig. 30.3). Within those nodules are a variant of RS cells called lacunar cells (so-called because they have smaller nuclei, less prominent nucleoli, and abundant cytoplasm). Unlike other forms of classical HL, this subtype shows a female predominance and is less associated with EBV infection. It has also been associated with high standard of living.

(b) *Mixed Cellularity*. It is the second most frequent subtype and represents about 10–25% of HL. It is more common in the developing countries where it is strongly associated with EBV infection. Furthermore, in contrast to NS, low socioeconomic status appears to be a risk factor. Younger children are also more frequently affected.

(c) *Lymphocyte-Rich*. Lymphocyte-rich histology represents about 5% of classical HL. Its features are intermediate between classical HL and NLPHL. Before advent of routine immunophenotyping, it was often misdiagnosed as NLPHL. It presents with early-stage disease and rarely involves the mediastinum.

(d) *Lymphocyte-Depleted*. It is the rarest form of HL. It shares overlapping features with mixed cellularity but is seen primarily in the older population. It is also associated with advanced disease, HIV infection, and underlying immunodeficiency states. Lymphocyte-depleted HL has the worst prognosis among all subtypes of HL.

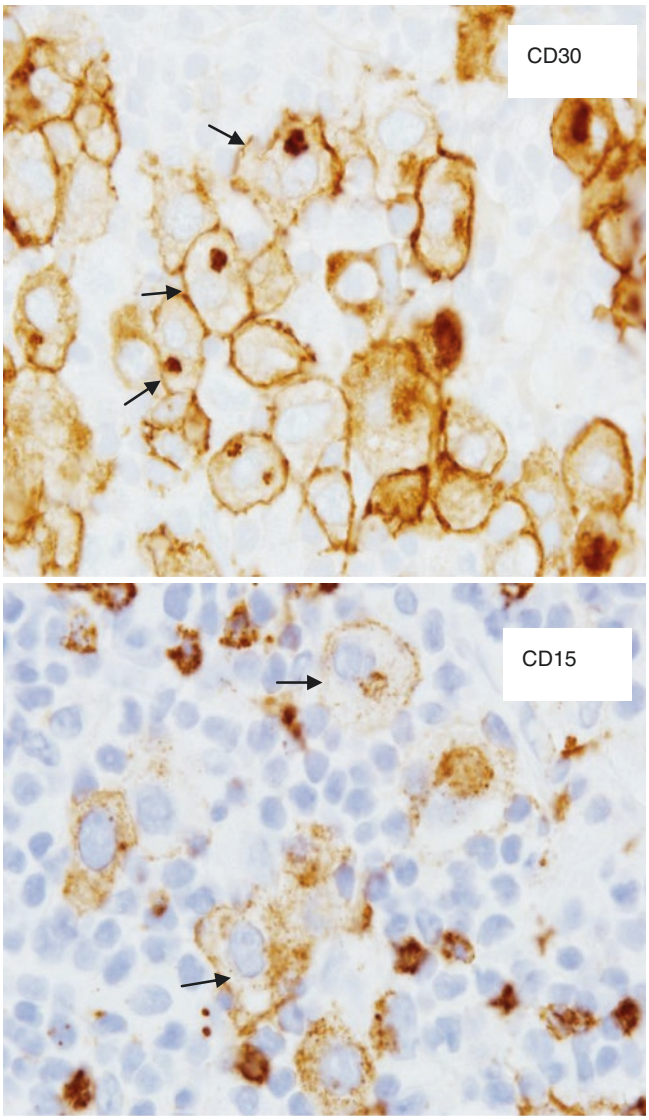

Fig. 30.2 CD30 and CD15 immunostains demonstrating Reed-Sternberg cells (arrows). (Image courtesy of Dr. S. Chen, Department of Pathology, Boston Children's Hospital, Boston MA)

2. *Nodular Lymphocyte-Predominant*. It constitutes about 5% of HL. RS cells are either lacking or absent. Instead, the disease is characterized by the presence of lymphocytic and histiocytic (L&H) cells (also called popcorn cells), recently reclassified as lymphocyte-predominant "LP" cells in the World Health Organization 2008 classification. In contrast to RS cells, these multinucleated LP cells have scanty cytoplasm with basophilic nucleoli. More importantly, unlike the RS cells, LP cells stain differently on immu-

Table 30.1 Histology subtypes of classical Hodgkin lymphoma

	Nodular sclerosis	Mixed cellularity	Lymphocyte- rich	Lymphocyte- depleted
Frequency	70%	20%	5%	<5%
Age	Young adults	Younger children	Young adults	Older adults
Sex	Female	Male	Male	Male
EBV association	Weak	Strong	Weak	Strong
Association with countries/regions	Developed countries	Developing countries	Developed countries	Developing countries
Presentation	Early-advanced stages	Early-advanced stages	Early stage	Advanced stage
Outcome	Very good	Very good	Excellent	Poorest

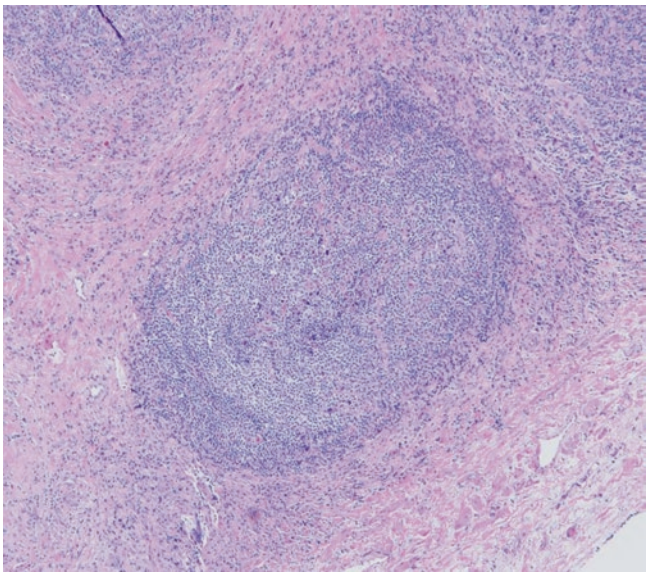

Fig. 30.3 H&E stain of nodular sclerosis histology. Dense inflammatory nodule in the center of image surrounded by fibrous collagen bands. (Image courtesy of Dr. S. Chen, Department of Pathology, Boston Children's Hospital, Boston MA)

nophenotyping and are CD20 positive and typically lack CD15 and/or CD30. NLPHL affects males predominantly and like lymphocyte-rich, in most cases presents with localized peripheral lymph node disease without mediastinal involvement. In addition, NLPHL has a higher tendency for late relapses and transformation to aggressive B-cell non-Hodgkin lymphoma.

Clinical Evaluation and Staging of Hodgkin Lymphoma

A. *Clinical Evaluation*
   1. *History*
      (a) Lymph node enlargement.
      (b) Fever. Rarely Pel-Ebstein cyclic fever is present. This refers to cyclic episodes of persistent high fevers alternating with periods of normal temperature.
      (c) Weight loss.
      (d) Drenching night sweats.
      (e) Pruritus.
      (f) Dyspnea and/or cough with bulky mediastinal involvement.
      (g) Facial swelling, fullness, or flushing in the setting of superior vena cava syndrome.
      (h) Alcohol-induced pain at the site of lymph node enlargement.
      (i) Rarely, paraneoplastic manifestations such as neurological symptoms.
   2. *Physical Exam*
      (a) Palpable painless lymphadenopathy.
      (b) Spleen and/or liver enlargement may be present.
      (c) Distended neck veins, facial swelling, or flushing in the setting of superior vena cava syndrome.
   3. *Differential Diagnosis*
      (a) Infections – Non-specific lymphadenitis, infectious mononucleosis, tuberculosis, cytomegalovirus, HIV, syphilis, cat scratch disease, and toxoplasmosis
      (b) Other malignancies – Non-Hodgkin Lymphoma, leukemia, and metastatic cancer
      (c) Connective tissue disease – Systemic lupus erythematosus, dermatomyositis, and scleroderma
      (d) Drugs – Phenytoin
      (e) Others – Sarcoidosis, Castleman's disease, and Kikuchi disease
   4. *Laboratory Studies*
      (a) Complete blood count which may demonstrate anemia, leukocytosis, neutrophilia, lymphopenia eosinophilia, or thrombocytosis.
      (b) Elevated erythrocyte sedimentation rate (ESR).
      (c) Serum lactate dehydrogenase (LDH) may also be elevated.
      (d) Decreased serum albumin and elevated alkaline phosphatase.
      (e) Baseline renal function.
   5. *Radiologic Studies*
      (a) Chest radiograph
      (b) Computed tomography (CT) scan of the chest, abdomen, and pelvis
      (c) 18-Fluoro-2-deoxy-D-glucose positron emission tomography (FDG-PET) scan

Table 30.2 The Ann Arbor staging classification

Stage[a]	Description[b]
I	Involvement of a single lymph node region (I) or a single extralymphatic organ or site (IE)
II	Involvement of two or more lymph node regions on same side of diaphragm (II) or local involvement of an extralymphatic organ or site and one or more lymph node regions on same side of diaphragm (IIE)
III	Involvement of lymph node regions on both sides of diaphragm (III), which may also be accompanied by involvement of the spleen (III_S) or by local involvement of an extralymphatic organ or site (IIIE) or both (III_SE)
IV	Diffuse or disseminated involvement of one or more extralymphatic organs or tissues, with or without lymph node involvement

Adapted and modified from Hoffman et al. [6]

[a]Letters following the Roman numerals are used to subclassify the stages. Fever >38 for more than three consecutive days, drenching night sweats, or unexplained loss of 10% or more of body weight in 6 months preceding diagnosis is denoted by B. A indicates absence of these symptoms. E indicates involvement of an extralymphatic site; S indicates spleen involvement

[b]X is designated if nodal mass >10 cm in maximum dimension or mediastinal mass >1/3 of chest on a chest X-ray

6. *Biopsies*
   (a) Diagnostic biopsy of affected lymph node
   (b) Bone marrow aspirate/biopsy in selected cases
   (c) Biopsy of suspicious disseminated extranodal sites (e.g., pulmonary or liver lesions) if clinically indicated

B. *Disease Staging*. The *Ann Arbor staging system* developed in 1971 is most widely used in the staging HL. It incorporates the extent of disease involvement or spread and the presence of specific systemic constitutional symptoms at diagnosis. The *Cotswold modification* to the Ann Arbor staging in 1988 included additional designations for the presence of bulky disease (defined as nodal mass greater than 10 cm in maximum dimension or mediastinal mass greater than 1/3 of chest on chest X-ray. See details of Ann Arbor staging in Table 30.2.

Treatment of Hodgkin Lymphoma

A. *Treatment of Newly Diagnosed Hodgkin Lymphoma*. The treatment of newly diagnosed HL has historically being guided by clinical stage (i.e., early or localized (Stage I/II) versus advanced stage (Stage III/IV). The different modalities used in treatment are discussed below. NLPHL is considered a distinct entity, and its treatment will be discussed separately.

1. *Radiotherapy*. Radiation therapy has been used to treat HL since the 1960s. It was initially the mainstay of treatment for patients with early-stage disease, and given concern for contiguity of spread of HL to adjacent lymph node groups, radiation was given to both clinically involved areas and the contiguous uninvolved nodal groups. This approach resulted in the development of the standard radiation fields, which have been further modified (Table 30.3) as a result of the long-term toxicities associated with radiation therapy (Table 30.4). Though it remains an important treatment modality, it is rarely used alone as treatment for classical HL but rather administered in smaller doses and volumes in combination with chemotherapy. Doses between 20 and 30 Gy are commonly used
2. *Chemotherapy*. The use of chemotherapy dates back to the discovery of nitrogen mustard in the early 1940s, but the first successful combination regimen evaluated in a clinical trial "MOPP" (mechlorethamine, vincristine, procarbazine, and prednisone) was developed about 20 years later. Since that time, different treatment regimens have been developed on this backbone by replacing alkylating agents (Table 30.5). The most commonly used chemotherapy regimen in HL is ABVD (doxorubicin {adriamycin}, bleomycin, vinblastine, and dacarbazine), which is administered in repeated cycles (typically a cycle lasting ~28 days with infusions on days 1 and 15). The number of recommended treatment cycles varies with type of regimen and stage of disease. In the adults, chemotherapy alone is the recommended treat-

Table 30.3 Radiation field designs used in Hodgkin lymphoma

Radiation field	Involved lymph nodes
Mantle	Submental, cervical, supra- and infraclavicular, axillary, hilar, and mediastinal lymph nodes
Mantle and paraaortic +/− splenic	Paraaortic lymph nodes in addition to the mantle field. The spleen is included in the treatment field in patients who do not undergo surgical staging and splenectomy. Also known as subtotal nodal or lymphoid irradiation
Total nodal irradiation	Most of the lympoid tissue, with the addition of a pelvic field to the mantle and paraaortic field. Does not include other nodal groups such as the brachial, epitrochlear, popliteal, sacral, and mesenteric nodes
Inverted-Y	Paraaortic and pelvic lymph nodes
Involved-field	Includes the involved lymph node region. These nodal regions are pre-defined. For example, a patient presenting with an enlarged cervical lymph node will receive irradiation to the entire ipsilateral cervical chain and the supraclavicular region, as these nodes are considered one region
Involved-site[a]	In contrast to involved-field, only the involved nodes (not nodal regions) are irradiated. Adjacent uninvolved nodal groups in the region are spared
Involved-node[a]	Similar to involved-site, the only difference is that it requires pre-chemotherapy PET imaging to have been performed in the radiation treatment position

[a]Preferred in the current management of HL

Table 30.4 Long-term sequelae of Hodgkin lymphoma therapy

Late toxicity	Agents/comments
Cardiac toxicity	Anthracyclines (doxorubicin), usually doses >300 mg/m^2
	Chest/mediastinal radiation
	Toxicity includes cardiomyopathy, coronary vascular disease, valvular disease, arrhythmias, and pericardial disease. Risk increases with combination of anthracycline and radiation
Secondary cancers	Leukemias/myelodysplastic syndrome – etoposide (early <3 years), cyclophosphamide (late >3 years) with MOPP and BEACOPP. ABVD is not associated with secondary MDS/AML
	Breast cancer – chest/mediastinal radiation
	Soft tissue sarcoma in radiation field
	Lung cancer, especially in smokers treated with radiation therapy
	Gastrointestinal cancers in the radiation field
	Thyroid cancer – neck radiation
Pulmonary dysfunction	Lung fibrosis/pulmonary insufficiency – bleomycin, chest irradiation
Hypothyroidism	Neck radiation
Infertility	Alkylating agents: nitrogen mustards (e.g., mechlorethamine), cyclophosphamide, procarbazine
	Pelvic radiation

ment for most patients with advanced HL. A recent randomized trial demonstrated that chemotherapy (AVD – doxorubicin, vinblastine, and dacarbazine) in combination with brentuximab, an antibody-drug conjugate targeting CD30, was associated with a slight improvement in progression-free survival compared to ABVD in patients with advanced disease. Bleomycin in ABVD was omitted due to increased pulmonary toxicity in combination with brentuximab. Like radiotherapy, chemotherapy maybe associated with significant long-term toxicities (Table 30.4). For patients, who fail primary chemotherapy, salvage regimens are listed in Table 30.6 (see below).

3. *Combined Therapy*. Combined modality treatment with chemotherapy and radiation therapy has been the mainstay of treatment for early-stage HL and has resulted in long-term survival exceeding 90%. Patients initiate treatment with chemotherapy and upon completion receive consolidative radiation therapy, allowing for smaller fields and less exposure to radiation. However even with lower doses and smaller fields of radiotherapy, long-term treatment-related toxicity remains a significant concern. Hence current treatment approaches and clinical trials focus on omission of radiation where possible and/or de-intensification of chemotherapy in a subset of patients with the most favorable disease (defined on basis on known prog-

Table 30.5 Examples of chemotherapy regimens used in newly diagnosed Hodgkin lymphoma

ABVD	Doxorubicin, bleomycin, vinblastine, and dacarbazine	Most common regimen
		Most preferred in adults
BEACOPP	Bleomycin, etoposide, doxorubin, cyclophosphamide, vincristine, procarazine, and prednisone	Given in escalated doses
		Compared with ABVD, escalated BEACOPP has superior progression-free survival rates but has increased toxicity and no difference in overall survival rates
Stanford V	Doxorubicin, vinblastine, mechlorethamine, vincristine, bleomycin, etoposide, and prednisone	Uses a brief intensive chemotherapy regimen combined with radiation to bulky lymph node sites
		The cumulative doses of doxorubicin, mechlorethamine, and bleomycin are reduced compared with ABVD and MOPP
MOPP	Mechlorethamine, vincristine, procarbazine, and prednisone	Less efficacy and greater toxicity compared to ABVD
		Rarely used in upfront treatment
ABVE-PC	Doxorubicin, bleomycin, vincristine, etoposide-prednisone, cyclophosphamide	More commonly used in pediatric centers in North America
OEPA-COPDAC	Vincristine, etoposide, prednisone, doxorubicin-cyclophosphamide, vincristine, prednisone, dacarbazine	Intensive regimen used in children and adolescents
		Common in Europe
VAMP	Vinblastine, doxorubicin, methotrexate, prednisone	Also used in some pediatric centers in North America

Table 30.6 Salvage chemotherapy regimens used in relapsed/refractory Hodgkin lymphoma

Salvage regimens	Chemotherapy agents
ICE	Ifosfamide, carboplatin, etoposide
DHAP	Dexamethasone, cytarabine, cisplatin
ESHAP	Etoposide, methylprednisolone, cytarabine, cisplatin
GVD	Gemcitabine, vinorelbine, liposomal doxorubicin
GDP	Gemcitabine, dexamethasone, cisplatin
IGEV	Ifosfamide, gemcitabine, vinorelbine, prednisone
Mini-BEAM	BCNU, etoposide, cytarabine, melphalan
Dexa-BEAM	Dexamethasone, BCNU, etoposide, cytarabine, melphalan

nostic risk factors) or who demonstrate a very favorable radiographic (typically by FDG-PET scan) early response to chemotherapy.

B. *Clinical Prognostic Factors in Hodgkin Lymphoma*. In addition to disease staging, the identification of poor risk factors in HL has been crucial in modifying current treatment paradigms. Continued concerns for treatment-related toxicities have informed the development of risk-based approach incorporating known or validated prognostic factors. These prognostic factors have been defined separately for early-stage (Table 30.7) and advanced-stage diseases (Table 30.8). Currently, the single most important tool for planning of treatment in HL is interim disease evaluation by FDG-PET. This response-based paradigm utilizing early response FDG-PET obtained usually after two cycles of chemotherapy (commonly referred to as PET-2) has been evaluated in numerous clinical trials and has gained acceptance among most oncology providers and treatment centers.

C. *Treatment of Nodular Lymphocyte-Predominant Hodgkin Lymphoma*. The majority of patients with NLPHL present with early-stage disease, and treatment with local radiation alone results in excellent outcomes and long-term survival. Watchful waiting and observation after surgical resection is also an option for some patients with completely resected Stage 1 disease. Although NLPHL distinctly expresses CD20, the use of rituximab, a chimeric anti-CD20 monoclonal antibody in upfront treatment of localized disease, is not widely recommended but may have a role in advanced-stage and relapsed disease. Similar to classical HL, the mainstay of treatment for advanced-stage NLPHL is combined chemotherapy. The optimal chemotherapy regimen has not been defined, and unlike with classical Hodgkin lymphoma, alkylating agents appear to be important.

D. *Treatment of Relapsed or Refractory Hodgkin Lymphoma*. Few patients will experience primary refractory or relapsed disease. For the majority of patients, treatment with alternative chemotherapy combinations (called second-line or salvage chemotherapy regimens) followed by high-dose chemotherapy and autologous hematopoietic cell transplant (AHCT) with or without consolidative radiotherapy has become the standard of care for relapsed/refractory HL disease in most centers. Autologous transplant is discussed in detail in Chap. 38. Apart from AHCT, novel drugs targeting specific proteins on the Reed-Sternberg cells have been developed. Two of such agents – brentuximab and PD-1 inhibitors – are also discussed below.

1. *Salvage Chemotherapy*. Achieving a complete remission to second-line chemotherapy by FDG-PET prior to ASCT is a strong predictor of long-term disease control. Multiple active chemotherapy regimens are available (Table 30.6).
2. *High-Dose Chemotherapy and AHCT*. High-dose chemotherapy is used as a preparative regimen before autologous transplantation. The goal of high-dose chemotherapy is to eliminate any residual malignant cells. The most common regimens used in HL are BEAM (BCNU, etoposide, cytarabine, and melphalan) and CBV (cyclophosphamide, BCNU, and etoposide). Many patients are successfully treated with high-dose chemotherapy and AHCT following salvage chemotherapy.

Table 30.7 Prognostic factors for early-stage HL according to European Organization for the Research and Treatment of Cancer (EORTC) and German Hodgkin Study Group (GHSG)

Group	Poor prognostic factors
EORTC	Age >50 years
	Bulky mediastinal mass (>1/3 maximum intrathoracic diameter)
	ESR >50 if no B symptoms present
	ESR >30 with B symptoms are present
	≥4 lymph node groups
GHSG	Bulky mediastinal mass (>1/3 maximum intrathoracic diameter)
	ESR >50 if no B symptoms present
	ESR >30 if B symptoms are present
	Presence of extranodal extension
	≥3 lymph node groups

Table 30.8 International prognostic score for advanced Hodgkin lymphoma

One point is given for each of the characteristics below present in the patient		
Serum albumin <4 g/dL		
Hemoglobin <10.5 g/dL		
Male gender		
Age >45 years		
Stage IV disease		
White blood cell count ≥15,000/μL		
Absolute lymphocyte count <600/μL and/or <8% of the total white blood cell count		
Score	**5-year freedom from progression**[a]	**5-year overall survival**[a]
0	84% (88%)	89% (98%)
1	77% (84%)	90% (97%)
2	67% (80%)	81% (91%)
3	60% (74%)	78% (88%)
4	51% (67%)	61% (85%)
5 or more	42% (62%)	56% (67%)

Adapted from Hasenclever et al. [14]. Copyright © 1998 *Massachusetts Medical Society. All rights reserved*

[a]Additional values in bracket are updated survival rates in patients treated with ABVD, from the British Columbia Cancer Agency Lymphoid Cancer Database (1980–2010) as reported by Moccia et al. [25]

3. *Novel Immunotherapy Agents*. Not all patients will respond to salvage chemotherapy, and some patients may relapse after AHCT. Many patients will be candidates for novel agents including immunotherapy.
   (a) *Brentuximab*. Brentuximab is an example of a class of drugs called antibody-drug conjugates (ADC) which are composed of monoclonal antibodies to specific tumor cell antigens that have been covalently linked to a separate anticancer agent. Brentuximab consists of an antibody against CD30, the transmembrane protein found on Reed-Sternberg cells, linked to the antineoplastic agent monomethyl auristatin E (MMAE). Binding of brentuximab to the tumor cell via the CD30 antigen allows internalization of MMAE which disrupts the organization of the microtubules in the tumor cell DNA, promoting cell death. This agent has been shown to be effective in patients with relapsed or refractory HL when used alone and in combination with other salvage agents. An example is the combination of brentuximab with bendamustine. Bendamustine belongs to a class of chemotherapy drugs called alkylating agents which act by cross-linking DNA. As single agent, bendamustine only resulted in modest improvement in disease-free progression in patients with relapsed/refractory HL. However in combination with brentuximab, most patients achieved complete disease remission and successfully proceeded to AHCT. Also recently, the combination of brentuximab with standard chemotherapy (AVD) was approved for upfront treatment in patients with newly diagnosed advanced disease. The drug is associated with peripheral neuropathy.
   (b) *PD-1 Inhibitors*. Tumor cells are generally able to evade the host immune surveillance system by overexpressing these certain cell surface proteins called "checkpoint proteins." One of such is the PD-1 ligands previously described. HL like many other cancers overexpresses PD-1 ligands. In addition, HL harbors genetic alterations that uniquely allow them to express these ligands. These antigens and their receptors are also expressed in normal immune cells and are necessary for maintenance of self-tolerance, and in their absence, autoimmunity can result. Recently, PD-1 inhibitors which are drugs that target PD-1 ligands (PDL-1 and PDL-2) have been used to treat patients with HL who had failed ASCT. PD-1 inhibitors include nivolumab and pembrolizumab. The role of these agents in HL is still emerging. As anticipated, rashes and autoimmune-like organ inflammatory states such as pneumonitis and colitis are frequently reported.
4. *Allogeneic Hematopoietic Stem Cell Transplantation*. Patients who relapse after AHCT or fail to respond to novel agents may be offered allogeneic hematopoietic cell transplant (HCT). Allogeneic HCT is discussed in Chap. 38.

Summary

The curability of Hodgkin lymphoma, a hematological malignancy arising from the lymphoid system, is one of the great successes of the twentieth century. Although excellent outcomes have been achieved through combination of chemotherapy and radiation therapy, this has been limited by long-term treatment toxicities. Therefore, current efforts have focused on reducing toxicity through both risk- and response-based treatment approaches. Patients who relapse or fail to respond to initial treatment modalities can be treated with salvage chemotherapy, followed by high-dose chemotherapy and autologous transplantation. The role of novel therapies like brentuximab and PD-1 inhibitors is evolving.

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Indolent Lymphomas

31

Thomas M. Habermann

Introduction

Small cell non-Hodgkin lymphomas are a pathologically distinct group of malignant disorders that arise from lymph nodes, the bone marrow and, in some histologies, in extranodal locations. Establishing the histopathological diagnosis through tissue biopsy is essential in predicting prognosis and treatment plans.

Distinctions Among the Indolent Lymphomas (Table 31.1)

Table 31.1 Types and frequencies of indolent lymphomas

Lymphoma subtype	Frequency
Follicular lymphoma	20%
Follicular lymphoma in situ	Up to 2% of reactive lymph nodes
Nodal marginal zone lymphoma	1.5%
Splenic marginal zone lymphoma	<1.5%
Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma	

A. Epidemiologic factors:
   (a) In an analysis of 596,476 patients from the National Cancer Database from 1998 to 2011 which covered 70% of US cancer cases, the prevalence of the major subtypes of small cell lymphomas was 17.1% in follicular lymphoma, 8.3% in marginal zone lymphoma, and 1.1% in lymphoplasmacytic lymphoma [3]. In these data set, the 5-year overall survival (OS) had improved over time. The incidence rates of all lymphoid neoplasms were 34.4 per 100,000 and age adjusted to the US general population in 2011 [4]. The incidence rates were as follows: follicular lymphoma (FL) 3.3, marginal zone lymphoma (MZL) 1.8, lymphoplasmacytic lymphoma (LPL) including Walenström macroglobulinemia (WM) 0.6, and primary cutaneous follicle center lymphoma 0.1.
   (b) The expected number of new cases by histology in 2016 was as follows: follicular lymphoma 13,960 (12%), marginal zone lymphoma 7460 (7%), and lymphoplasmacytic lymphoma 2330 (2%) [4].
   (c) The incidence of FL and MZL increase from age 30 to age 70 years.
   (d) In contrast to other NHL subtypes, there is not a male dominance in FL and MZL. With regard to etiology, Sjögren's syndrome is a risk factor in FL, MZL, and LPL/WM [5]. There is an association of hepatitis C in MZL and LPL/WM. The risk of FL is increased with a first-degree relative with non-Hodgkin lymphoma. In MZL, *Helicobacter pylori* are associated with gastric lymphoma. Hepatitis C virus has a role in some types of MZL. Decreased risk was associated with alcohol consumption in MZL and LP/WM in a study of 17,471 cases and 23,096 controls. Incidence rates for FL, MZL, and LPL lymphomas are lower in among blacks and Hispanics.
   (e) In contrast to aggressive lymphomas, the 2-year survival rates range from 89% to 95% in MZL and 88–93% in FL.

B. Clinical presentations:
   (a) Lymphadenopathy:
      (i) The most common presentation is asymptomatic lymphadenopathy.
      (ii) There are over 150 causes of lymphadenopathy including cancer, hypersensitivity syndromes, infection, connective tissue diseases, atypical lymphoproliferative disorders, granulomatous disorders, and other unusual causes of lymphadenopathy (acronym CHICAGO) [6].
      (iii) The lymphadenopathy is usually painless but may be painful.

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(b) Extranodal involvement is a common characteristic in marginal zone lymphoma with sites that include gastric, parotid, pulmonary, and other locations.
(c) "B" symptoms or systemic constitutional symptoms include fever without infection, night sweats, and unexplained weight loss of greater than 10% of body weight in less than 6 months can be present in indolent lymphomas, but these are more common in aggressive lymphomas.

C. Staging:
(a) The Ann Arbor staging system is used for indolent non-Hodgkin lymphoma.
(b) Patients are sub-classified to indicate the absence (A) or presence (B) of constitutional symptoms.
(c) The methods to stage indolent non-Hodgkin lymphoma are similar to other lymphomas. These are used to determine the overall stage and identify prognostic factors that may influence the management and outcome of indolent lymphoma.
(i) History:
1. Unexplained fever
2. Weight loss
3. Night sweats
4. Fatigue
5. Pain
6. Performance status:
(a) This is an assessment of the patient's daily activities that are either a reflection of the amount of disease that the patient has or would be a reflection of other comorbidities that would potentially impact tolerance to treatment and ultimately influence prognosis and overall survival.
(b) The most commonly incorporated scale is the Eastern Cooperative Oncology Group (ECOG) scale:
(i) 0 = asymptomatic, fully active
(ii) 1 = symptomatic but completely ambulatory
(iii) 2 = symptomatic but in bed <50% of the time
(iv) 3 = symptomatic and in bed >50% of the time
(v) 4 = bedbound, completely disabled
(ii) Physical examination:
1. Palpable lymphadenopathy
2. Size of the liver and spleen
3. Palpable parotid gland
(iii) Laboratory studies:
1. Complete blood count, differential
2. Serum alkaline phosphatase, lactic dehydrogenase, albumin, uric acid, calcium
3. Renal function (creatinine, blood urea nitrogen [BUN])
4. Liver function studies
5. Beta-2 microglobulin (in follicular lymphoma)
6. Hepatitis B and C serologies
7. HIV serologies
(iv) Radiologic studies:
1. Computed tomography (CT) scan of the chest, abdomen, and pelvis
2. Positron emission tomography (PET) scan
(v) Biopsies:
1. Diagnostic biopsy of affected lymph node
2. Bone marrow biopsy
3. Biopsy of suspicious extranodal sites (e.g., pulmonary, parotid, gastric, etc.) if clinically indicated

D. Pathology (excluding pediatric types). The diagnosis requires a tissue diagnosis.
(a) Follicular lymphoma:
(i) Follicular lymphoma:
1. Follicular grades 1, 2, and 3A are considered non-aggressive.
2. Follicular grade 3B should be treated as diffuse large B-cell lymphoma (DLBCL).
(ii) In situ follicular neoplasia
(iii) Duodenal-type follicular lymphoma
(b) Marginal zone lymphoma:
(i) Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma)
(ii) Splenic marginal zone lymphoma
(iii) Nodal marginal zone lymphoma
(c) Lymphoplasmacytic lymphoma/Waldenström macroglobulinemia (LPL/WM)
(d) Primary cutaneous follicle center lymphoma

Treatment

A. Follicular lymphoma (FL): The overall survival has improved in patients with FL over the last two decades.
(a) Follicular lymphoma, grades 1, 2, and 3A:
(i) Stage I:
1. Radiation therapy
(a) Limited-stage FL is the only potentially curable stage FL.
2. Observation
(ii) Stages II–IV:
1. Observation ("watch and wait"): Asymptomatic patients with non-bulky disease, with no threatened end-organ function, not steadily progressing or cytopenias may

be observed. The most commonly employed criteria for treatment are the GELF (Groupe D'Etude des Lymphomes Folliculaires) which are low-tumor burden as defined as no mass >7 cm, <three masses >3 cm, no systemic or B symptoms, no splenomegaly >16 cm by CT scan, no risk of vital organ compression, no leukemic phase >5000/μL circulating lymphocytes, and no cytopenias (defined as platelets <100,000/μL, hemoglobin <10 g/dL, or absolute neutrophil count <1500/μL) [7]. Initial observation is supported by the National Comprehensive Cancer Network (NCCN) guidelines, the British Society of Hematology, and the Lymphoma Canadian Scientific Advisory Committee. Patients who progress, relapse, or are re-treated within 12 months from the time of diagnosis have a modest increase in mortality compared to the general population, while those that achieve an event-free survival at 12 months (EFS12) after initial observation have an excellent subsequent prognosis as related to sex- and age-matched population data [13].

2. Single-agent rituximab is the initial treatment of choice in low-tumor burden patients. The majority of patients with FL present with low-tumor burden.
   (a) In a trial by Ardeshna and colleagues comparing observation versus four weekly doses of rituximab versus four weekly doses of rituximab followed by maintenance therapy every 2 months for 2 years, 60% of patients with four doses of rituximab remained progression-free at 3 years with a 3-year overall survival (OS) of 94% in the observation arm and 97% in the maintenance arm [8]. In this study, there was a significant difference in time to start of a new treatment with 54% of the patients in the observation being treated by 3 years in contrast to 12% of patients in the group that received rituximab followed by maintenance rituximab.
   (b) In the RESORT trial, patients were initially treated with single-agent rituximab at a dose of 375 mg/m^2 for four doses and then randomized to maintenance rituximab or observation with re-treatment at each disease progression [9]. In 289 patients, the estimated time to treatment failure was 3.9 years in the re-treatment arm and 4.3 years in patients treated with maintenance rituximab (P = 0.54). The median number of rituximab doses was 4 in patients in the re-treatment arm and 18 for those in the maintenance rituximab arm. There were no differences in anxiety in either treatment approach [10].
   (c) In the National LymphoCare database, 15–20% of patients with FL were treated with single-agent rituximab as their initial therapy [11].
   (d) Patients failing to achieve an event-free survival at 12 months (EFS12) with single-agent rituximab had a small number of increased FL deaths. Monotherapy with short-course rituximab provides prolonged disease-free survival with minimal toxicity [13].
3. Immunochemotherapy: The most common immunochemotherapy regimens employed internationally are R-CVP (rituximab, cyclophosphamide, vincristine, and prednisone), R-CHOP (rituximab, cyclophosphamide, Adriamycin [doxorubicin], vincristine [Oncovin], and prednisone), and BR (bendamustine/rituximab). BR has been more commonly utilized in recent years with less alopecia, infections, and peripheral neuropathy than R-CHOP therapy and an improved progression-free survival (PFS). The OS in FL has improved in the immunochemotherapy era with recent estimates from the National LymphoCare study describing 70–80% OS rates at 8 years [11, 14].
4. Early event status, as defined as time from diagnosis to progression, relapse, re-treatment, or death due to any cause, is predictive of outcome in FL [13]. Patients who fail to achieve an event-free survival at 12 months (EFS 12) and 24 months (EFS24) have an inferior OS. In contrast, patients achieving EFS12 have no added mortality beyond the age- and sex-matched population. Patients with early events after immunochemotherapy have especially poor outcomes. Reassessment of patients status at 12 months from diagnosis in FL patients or at 24 months in patients treated with immunochemotherapy is a strong predictor of subsequent OS in FL. The 2-year OS rates of the EFS12 failures were in the range of 78% versus 98% in those

patients who achieved EFS12. The median survival of these EFS12 failures was approximately 3 years versus 97% in those patients who were event-free.

5. Transformation: Follicular lymphoma may transform into diffuse large B-cell lymphoma. Historically the rates of transformation were 3–4% per year with 5-year survival rates of 17–21%. The risk of transformation in a population-based study by Link et al. was 10.7% at 5 years, with an estimated rate of transformation of 2% per year [12]. The transformation rate was highest in patients who were initially observed and lowest in patients who initially received rituximab monotherapy (14.4% *v* 3.2%; $P = 0.021$). The median OS following transformation was 50 months and was superior in patients with transformation greater than 18 months after FL diagnosis compared with earlier transformation (5-year overall survival 66% versus 22%; $P < 0.001$). In this report, previously anthracycline-naïve patients who receive R-CHOP after transformation had outcomes indistinguishable from patients with de novo DLBCL. Hematopoietic cell transplantation is a consideration in these patients. In a multicenter analysis of 172 patients with transformed FL, there were improved outcomes in patients treated with autologous hematopoietic cell transplantation [15].
6. Hematopoietic cell transplantation: Autologous hematopoietic cell transplantation (AHCT) and allogeneic hematopoietic cell transplantation have been incorporated into the management of patients with relapsed or refractory disease. The risk of graft versus host disease and the expected 25% mortality rates in the first 100 days preclude widespread use of allogeneic transplant approaches in FL. The use of high-dose therapy with AHCT in the treatment of FL has not been fully established. A meta-analysis reported that high-dose therapy and AHCT do not improve OS in FL [16]. Therefore, the upfront use of AHCT should be in clinical trials and in selected patients. The International Bone Marrow Transplant Registry reported on 904 patients with FL. In 176 allogeneic HCT, 131 purged AHCT, and 597 unpurged transplants, the recurrence rates were 21%, 43%, and 58% with 5-year OS rates of 51%, 62%, and 55% [17].
7. New agents:
   (a) Second-generation anti-CD20 antibodies are under evaluation. Obinutuzumab, a second generation type 2 anti-CD20 antibody has a 50% overall response rate [18]. With combination chemotherapy, progression-free survival rates are superior. In the GADOLIN study, patients refractory to rituximab were randomized to obinutozumab plus bendamustine or bendamustine monotherapy with non-progressing patients in the obinutuzumab group going on the maintenance obinutuzumab [19]. The progression-free survival was improved in patients treated with bendamustine and obinutuzumab.
   (b) Lenalidomide is an immunomodulatory agent. The overall response rate (ORR) in the relapsed/refractory setting was approximately 30% [20, 21]. When combined with rituximab, the CR rate was 87% which has led to a multicenter phase III open label randomized study comparing lenalidomide and rituximab versus rituximab chemotherapy followed by rituximab maintenance in previously untreated patients (the "RELEVANCE" trial).
   (c) Ibrutinib as a single agent had ORR of 20–30%. Combined with rituximab in the frontline setting, the ORR was 75–85% in two different arms [22].
   (d) Venetoclax is an inhibitor of BCL2. The ORR rate is 38% as a single agent, and combination trials are under way with BCL2 inhibitors [24].
   (e) Immune checkpoint inhibition drugs are being evaluated. In relapsed FL, pembrolizumab had an ORR of 66% and a CR rate of 52% with response duration of 18 months. Studies with nivolumab, durvalumab, and atezolizumab are ongoing.
8. Radiation therapy:
   (a) Full-dose radiotherapy and reduced-dose radiotherapy for local control with ORRs of 93% and 92% have been reported in a randomized study.
9. Future studies in the biology of FL will lead to improved understanding of factors that relate to outcome. FL is caused by a t(14;18) chromosomal translocation that juxtaposes the *BCL2* gene and immunoglobulin locus. *BCL2* coding sequence mutations correlate

with the risk of transformation and increase risk of death due to lymphoma [26].

(b) In situ follicular neoplasia:
  (i) Observation. This is considered a precursor lesion with low risk of progression.

(c) Duodenal-type follicular lymphoma:
  (i) Observation. This is a localized variant usually in the second portion of the duodenum with a low risk of dissemination. There are few randomized studies to guide treatment approaches. The treatment approaches are very heterogeneous.
  (ii) Gastric:
    1. The National Comprehensive Cancer Network (NCCN) and the European Society of Medical Oncology (ESMO) have published practice guidelines for diagnosis, treatment, and follow-up [27].
    2. Antimicrobial therapy: *H. Pylori* eradication (HPE) with triple therapy (proton pump inhibitor, amoxicillin, and clarithromycin) or quadruple therapy (proton pump inhibitor, bismuth, tetracycline, and metronidazole) are the standard therapies for gastric MALT irrespective of stage. Seventy-five to 85% of patients respond to the antimicrobial therapy. With long-term follow-up, 30% of patients are in complete remission (CR), and 60% of patients remain stable for years. In a meta-analysis of 1408 patients, the ORR for stage IE disease was 78% [28]. The 5-year OS was 90%, and the 5-year DFS was 75%. The rate of HP-negative cases appears to be increasing over time, from 5–10% to 35–50%. Recent guidelines advocate antibiotic therapy as the sole initial management in HP-negative patients. The reason for this is unclear, but one explanation is that clarithromycin has an antineoplastic mechanism of action. Absence of HP, mucosal invasion, and t(11;18)/*AP12/MALT1* are independent predictors of resistance to antimicrobial therapy. Responses to antimicrobial therapy may be delayed, and repeat biopsies should be performed no sooner than 3 months after antimicrobial therapy. Once the endoscopy is normal, patients can be observed with no follow-up endoscopies.
    3. Involved field radiation therapy (RT):
      (a) The CR rate with RT was 100% with an event-free survival of 100% at a median follow-up of 27% [29]. This is the treatment of choice in patients who have relapsed after antibiotic therapy.
    4. Rituximab:
      (a) The ORR after HPE therapy with single-agent rituximab was 77% with a CR rate of 46% with 54% of patients disease-free [30]. There were no differences in outcomes based on t(11;18) status.
    5. Immunochemotherapy:
      (a) The approaches to FL are the same approaches and are supported by the NCCN Guidelines.
  (iii) Pulmonary:
    1. Pulmonary marginal zone lymphoma is most commonly limited stage with only 14% of patients in the largest published series having stage III/IV disease [31]. Forty-five percent of patients had a history smoking in the International Extranodal Lymphoma Study Group (IELSG) study of 205 patients.
    2. In a retrospective report of 205 patients from 17 centers, the 10-year overall survival was about 75% [31].
    3. Treatment approaches include observation, surgery to remove the lesion of a single site, single-agent rituximab, and systemic chemotherapy.
    4. Observation or a "watch and wait" approach may be incorporated in primary pulmonary lymphoma at the time of initial diagnosis or in asymptomatic patients who have relapsed.
    5. Surgical resection: In patients managed with a local approach, mainly surgical resection, there was an improved progression-free survival versus those receiving systemic treatment in the IELSG study where 63 (30%) of patients underwent surgical resection of the disease and experienced an improved PFS ($p = 0.003$) [31].
    6. Rituximab monotherapy: Only 20 patients in the IELSG data set were treated with rituximab monotherapy [31]. The ORR was 40%.
    7. Systemic treatment approaches include single-agent rituximab or immunochemotherapy which would include BR, R-CVP, or R-CHOP as in the FL treatment. The PFS at 5 years in the IELSG study was 65% [31]. Systemic treatment is reserved for patients with advanced disease, patients who are in relapse after incomplete surgical resection, and patients who have relapsed.
    8. Patients who relapse with non-bulky disease and who are asymptomatic may be observed.

(iv) Parotid:
1. Patients with parotid marginal zone lymphoma present with parotid gland swelling. In the IELSG 41 study of 247 patients, 76% presented with limited-stage disease [32]. Local lymph nodes and the contralateral parotid gland may be involved.
2. MZL of the parotid glands was associated with autoimmune disease in IELSG 41 in 41% of patients [32]. Sjögren's syndrome was the most common occurring in 83% of patients. Of interest, Sjögren's syndrome is associated with an improved OS in MZL.
3. Patients with parotid gland marginal zone lymphoma have a long survival. The median survival was 18.3 years in the IELSG 41 study, and the median time to progression was 9.3 years [32].
4. Patients with salivary gland MALT lymphoma have an excellent prognosis regardless of initial treatment. Treatment approaches include the following:
   (a) Observation
   (b) Surgery:
      (i) The major morbidities to this approach are facial nerve injury and paralysis, scarring, sensory loss, auriculotemporal nerve syndrome, wound infection, sialocele, and fistula.
      (ii) In IELSG 41, 81 patients were treated with surgery and 30 had combined radiation therapy [32].
      (iii) In IELSG 41, there was no difference in OS or PFS for those patients who underwent complete excision ($n = 59$) versus those who underwent a partial resection ($n = 33$).
   (c) Radiation therapy:
      (i) The major morbidities to this approach include xerostomia, altered appetite, impaired swallowing, and dental caries.
   (d) Combined surgery and radiation therapy
   (e) Immunochemotherapy:
      (i) Patients whose initial therapy included rituximab had an increased OS (median not yet reached, $p = 0.03$) in the IELSG 41 study.

(v) Ocular lymphoma:
1. The incidence of ocular lymphoma is remarkably variable in different countries. *Chlamydophila psittaci* (CP) has been reported to be associated with orbital marginal zone lymphomas. Response rates to antibiotic therapy have ranged from 0% to 100% with an average of 23%. Both CP-positive and CP-negative patients have been reported to respond to antibiotic treatment with reported regression rates of 0 to the range of 33–65%.
2. Radiation therapy will potentially eradicate this disease. Reduced-dose radiation therapy should be considered [25].

(vi) Other sites with incidence rates of less than 4%: colon, thyroid, and spleen.

(d) Splenic marginal zone lymphoma:
   (i) This accounts for 20% of MZL cases.
   (ii) In patients with significant splenomegaly, splenectomy is the treatment of choice, and patients experience a prolonged disease-free survival. This treatment can be performed safely and offers durable long-term remissions in a number of patients [33].
   (iii) Anti-HCV therapy.

(e) Nodal marginal zone lymphoma:
   (i) Nodal marginal zone lymphoma accounts for 10% of MZL cases.
   (ii) The 5-year OS was 56% in nodal marginal zone lymphoma as opposed to 81% in MALT lymphoma with respective 5-year failure-free survival rates of 28% and 56%.
   (iii) The treatment of nodal marginal zone lymphoma is according to FL algorithms which is the current recommendation of the NCCN Guidelines.
   (iv) Initial management strategies include observation, single-agent rituximab, and immunochemotherapy.
   (v) Single-agent rituximab. In a study that randomized patients to maintenance rituximab versus observation in the ECOG trial E4402, there was an improvement in the time to treatment failure in patients who were treated with rituximab 375 mg/M^2 weekly times four followed by maintenance rituximab every 3 months until treatment failure [34].
   (vi) Immunochemotherapy. In patients with advanced disease, immunochemotherapy is the treatment of choice.
   (vii) Anti-HCV therapy.
   (viii) The transformation rates are 12.5–18%, and these patients should be managed in similar treatment patterns as DLBCL.

B. Lymphoplasmacytic lymphoma/Waldenström macroglobulinemia (LPL/WM):

(a) LPL/WM is not curable and has a heterogeneous course.
(b) Waldenström macroglobulinemia must be differentiated from an IgM monoclonal protein of undetermined significance (MGUS) and smoldering LPL/WM.
(c) Greater than 90% of LPL/WM harbor the *MYD88* L265P genetic mutation. This mutation is not specific to LPL/WM as 50% of IgM MGUS, 30% of diffuse large B-cell lymphoma of non-germinal center type, 50% of primary cutaneous DLBCL, leg type, and some cases of nodal and splenic marginal zone harbor the mutation.
(d) Treatment approaches [35]:
    (i) Observation: Patients with IgM MGUS or smoldering LPL/WM with preserved marrow function should be observed with follow-up at 6 months initially and then annually with a history, physical, and laboratory tests. Patients with smoldering LPL/WM require lifelong active surveillance, every 4 months for the first 3 years, every 6 months for the subsequent 2 years, and, if stable, annually thereafter.
    (ii) Single-agent rituximab: The median PFS is 16–29 months with an ORR of 25–40%. Rituximab monotherapy is contraindicated in patients with symptomatic hyperviscosity and is best avoided in patients with very high serum IgM levels.
    (iii) Plasma exchange should be urgently initiated before immunochemotherapy for hyperviscosity-related symptoms which include spontaneous bleeding from mucous membranes, visual changes from retinopathy, and neurologic symptoms (ranging from headache and vertigo to seizures and coma).
    (iv) Immunochemotherapy:
        1. BR is recommended for bulky disease, significant cytopenias, and constitutional symptoms.
        2. Dexamethasone-rituximab-cyclophosphamide (DRC) is an alternative in non-bulky disease.
    (v) Hematopoietic cell transplantation:
        1. Hematopoietic cell harvest for future use in patients aged 70 years and younger who are potential candidates for AHCT
    (vi) Treatment of patients in relapse:
        1. The original therapy may be considered if the time to next therapy is ≥3 years.
        2. In patients harboring *MYD88* L265P mutation, ibrutinib is efficacious [36].

C. Primary cutaneous follicle center cell lymphoma:
(a) The differential diagnosis of primary cutaneous lymphomas includes primary cutaneous follicle center cell lymphoma, marginal zone lymphoma, and primary cutaneous DLBCL lymphoma, leg type.
(b) This is a localized disease of the skin and management should reflect this.
    (i) Observation
    (ii) Single-agent rituximab
    (iii) Local radiation therapy

Summary

The spectrum and management decisions in the small B-cell lymphomas have become more complex over time. Rituximab has improved outcomes in this group of small B-cell lymphomas. Patients can live for many years with these chronic diseases.

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Aggressive Lymphoma

32

Timothy Fenske and Jonathan T. Kapke

Introduction

Non-Hodgkin lymphoma (NHL) is a heterogeneous group of hematologic malignancies derived from lymphocytes. The normal counterpart of the malignant cell can be of B- or T- or NK-cell origin and from various stages in differentiation (mature or immature). Clinically, it is useful to classify NHLs as either aggressive or indolent, since these two categories differ in terms of natural history, prognosis, potential for cure, and treatment strategies. In this chapter, we will present the epidemiology, diagnosis, staging, clinical presentation, prognostication, and treatment of the most common subtypes of aggressive B- and T-cell non-Hodgkin lymphoma.

Epidemiology and Classification

According to the Surveillance, Epidemiology, and End Results (SEER) Program database, an estimated 72,580 new cases of NHL were diagnosed in 2016, with 20,150 estimated deaths. NHL is estimated to account for 4.3% of all new cancer diagnoses and 3.4% of all cancer deaths in the year 2016. Over the past 10 years, the overall incidence of NHL has not increased; however, death rates for NHL have declined by 2.4% per year between 2004 and 2013 [1].

Several B-cell NHL subtypes are considered to be clinically aggressive. The most common type of aggressive B-cell NHL is diffuse large B-cell lymphoma (DLBCL). DLBCL accounts for approximately 30% of all NHL diagnosed annually in the USA [2]. Other aggressive B-cell NHL subtypes that will be discussed include mantle cell lymphoma, Burkitt lymphoma, transformed lymphoma, and high-grade B-cell lymphoma with MYC and BCL2 and/or BCL6 rearrangements (commonly known as "double-hit" or "triple-hit" lymphoma). "Double-hit" lymphoma refers to aggressive B-cell NHLs with translocation of c-MYC as well as a translocation of BCL2 or BCL6. "Triple-hit" lymphoma refers to the presence of all three rearrangements. Myc rearrangement and "double-hit"/"triple-hit" lymphoma have been associated with inferior outcomes with standard chemotherapy.

Most mature peripheral T-cell lymphomas (collectively referred to as "PTCL") subtypes also have aggressive features. The most common subtype of PTCL is PTCL-not otherwise specified (PTCL-NOS). Other common subtypes include angioimmunoblastic T-cell lymphoma and anaplastic large cell lymphoma. These three subtypes account for approximately 60% of all PTCL [3]. The incidence of PTCL is approximately <1 per 100,000 person-years, and it varies by ethnicity and region [4]. PTCL is more common in Asia where it is estimated to comprise up to 15–20% of all NHL [3]. NK-/T-cell lymphoma is also much more common in Asia. In the USA, the incidence of PTCL is highest in Blacks and lower in Caucasians, Hispanics, Asians, and Native Americans [5]. Potential risk factors include infectious hepatitis, autoimmune disease, and certain environmental exposures such as benzene, herbicides, and radiation.

The immature (precursor) B- and T-cell neoplasms, such as B- and T- lymphoblastic leukemia/lymphoma, are clinically aggressive. These malignancies usually have a leukemic component and rarely present as pure lymphomas. Such cases are therefore typically approached using acute lymphoblastic leukemia (ALL) treatment strategies. As a result, and due to their rarity in presentation as a lymphoma, we will not discuss these entities further and will instead focus on mature B- and T- cell neoplasms in adults.

Clinical Presentation

The clinical presentation of aggressive lymphoma is highly variable, based on the specific subtype and the location of involvement. Aggressive lymphomas, when compared to

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more indolent subtypes, more frequently present with rapidly growing masses, B symptoms (fevers, drenching night sweats, and weight loss) as well as laboratory abnormalities such as elevated lactic dehydrogenase (LDH) and other electrolyte abnormalities such as elevated uric acid related to tumor lysis. However, in contrast to the generally incurable indolent lymphomas, aggressive lymphoma can often be cured with standard treatment.

During initial presentation and workup, one should be aware of the potential oncologic emergencies related to aggressive NHL. Specific clinical situations that require immediate recognition and intervention include spinal cord compression, superior vena cava (SVC) syndrome, pericardial tamponade, hypercalcemia, tumor lysis syndrome, obstructive uropathy, and bowel obstruction or perforation. Aggressive diagnostic, prophylaxis and treatment measures should be pursued if these clinical situations are suspected or diagnosed.

In most cases the clinical presentation is much less dramatic, such as painless adenopathy, back pain, vague abdominal symptoms, fatigue, or lower extremity swelling. NHL can mimic many other conditions and require extensive workup. It is not uncommon for patients to undergo evaluation by specialists in several different fields, for weeks or months, before the diagnosis of lymphoma is finally made.

Diagnosis

Accurate and rapid pathologic diagnosis should be the primary objective for patients who present with clinical features suggesting an aggressive lymphoma. Obtaining an appropriate tissue biopsy is of utmost importance. Historically, an incisional or excisional lymph node biopsy has been considered the gold standard for tissue diagnosis in NHL. A surgical biopsy remains the most reliable approach to ensure that adequate tissue is obtained for pathology and other ancillary studies. However, a generous core needle biopsy specimen can be sufficient, particularly in cases where the lesion of interest would require an extensive or high-risk surgical procedure. Therefore, it is reasonable to attempt a large gauge core needle biopsy first in such cases, with the understanding that a surgical biopsy may ultimately still be necessary. A fine needle aspiration (FNA) alone is *not* sufficient to establish a diagnosis of lymphoma.

To differentiate between various NHL subtypes, immunophenotyping using immunohistochemistry and/or flow cytometry is routinely used. In addition, genetic testing with conventional metaphase cytogenetic analysis (karyotyping) and/or fluorescent in situ hybridization (FISH) is important to establish the diagnosis in some cases of NHL. Molecular testing using various techniques may also be important to help clarify certain subtypes of NHL and is expected to play a larger role in the coming years in terms of prognosis and selection of therapy. *It is therefore critical that the surgeon or radiologist performing the biopsy procedure consult with the hematologist/oncologist and/or pathologist to ensure that the diagnostic material is submitted properly.* For example, flow cytometry and metaphase cytogenetics cannot be performed if all of the diagnostic material is placed in formalin.

Initial Workup and Staging

The initial workup for patients with a newly diagnosed aggressive NHL starts with a detailed history and physical exam. History should include a complete review of systems and investigation of B symptoms including fever, drenching night sweats, or weight loss (Table 32.1). Physical exam should incorporate detailed evaluation of palpable adenopathy (location and size) spleen size and examination of pertinent systems guided by the patient's symptoms. A full

Table 32.1 Approach to workup, diagnosis, and staging for aggressive NHL

History
Emphasis on B symptoms and performance status
Infectious hepatitis
Autoimmune disease
Environmental exposure
Complete review of systems
Physical exam
Particular attention to nodal areas, the liver and spleen
Examination should also be guided by clinical history and other diagnostic data
Laboratory testing
CBC with differential
CMP
LDH
Hepatitis B, C testing
Pregnancy test if of childbearing age
HIV testing and beta-2-microglobulin
Imaging
PET/CT to complete staging
Echocardiogram or MUGA if anthracycline-containing regimen considered
CT head or MRI brain should be considered especially if CNS disease suggested by H&P
Procedures
Bone marrow aspiration and biopsy
Lumbar puncture
Fertility assessment and potentially sperm banking or egg storage
Prognostic evaluation
IPI (or other prognostic index depending on histologic subtype) should be calculated at diagnosis

From [8, 60]

neurological exam (with special attention to cranial nerve exam) is important as well to assess for possible central nervous system involvement by NHL. Laboratory testing should include complete blood count (CBC) with differential, lactate dehydrogenase (LDH), comprehensive metabolic panel (CMP), uric acid, hepatitis B and C serological testing, pregnancy testing if of childbearing age, and HIV screening. Imaging with an echocardiogram or multigated acquisition (MUGA) scan should be obtained if anthracycline-based chemotherapy is planned. Additionally, head CT or magnetic resonance imaging (MRI) of the brain should also be considered, particularly if there are signs or symptoms concerning for involvement of the central nervous system (CNS). Depending on the lymphoma subtype and clinical presentation, other procedures that may be indicated during initial evaluation include bone marrow aspiration and biopsy, lumbar puncture (with cytology and flow cytometry), and upper and/or lower GI endoscopy. Patients should be offered fertility evaluation with sperm banking or egg/embryo storage, when feasible.

Positron emission tomography with contrast-enhanced computed tomography (PET/CT) is the preferred imaging modality at initial staging for patients with newly diagnosed aggressive NHL. PET/CT has a high sensitivity and moderately high specificity when used for staging lymphoma [6]. PET/CT is typically preferred over routine contrast-enhanced computed tomography (CT) for traditionally FDG-avid lymphomas due to a higher sensitivity, particularly for certain extranodal sites of disease (bone, skin, soft tissue) that do not image as well with CT [7]. In addition, PET/CT can detect non-enlarged yet FDG-avid sites of nodal disease which would be missed on CT. PET/CT also has prognostic value when performed after therapy administration (either partway through therapy or at the end of therapy). Disadvantages of PET/CT include an increased risk for false-positive findings leading to unnecessary additional procedures and limited ability to detect lesions <1 cm in size, particularly in certain locations such as the lungs. However, PET/CT remains the recommended imaging modality for staging of traditionally FDG-avid lymphoma subtypes. This would include all NHL subtypes considered to be aggressive [8]. There may also be additional value in obtaining a contrast-enhanced diagnostic CT scan of the neck, chest, abdomen, and pelvis in certain cases, particularly when unusual sites are involved by lymphoma.

Staging for patients with newly diagnosed NHL is typically based on the location and extent of both lymph node and extralymphatic involvement. The most commonly used staging system is the Lugano Classification which is a modification of the Ann Arbor staging system originally developed for Hodgkin lymphoma (HL) [9]. The Lugano Classification incorporates PET/CT scan results into staging (Table 32.2).

Table 32.2 Lugano modification of Ann Arbor staging system

Stage	Definition
I	One node or group of adjacent nodes
II	Two or more nodal groups on the same side of the diaphragm
III	Lymph node involvement on both sides of the diaphragm
IV	Presence of diffuse or disseminated involvement of one or more extralymphatic organs with or without lymph node involvement (examples include the liver, lung, or bone marrow)
Subscript	**Definition**
X (bulky)	Typically defined as solitary mass >10 cm
E	Limited contiguous extranodal involvement
B	Presence of B symptoms (fever, night sweats, weight loss)

From [9]

Aggressive B-Cell Lymphoma Subtypes

Diffuse Large B-Cell Lymphoma (DLBCL)

Diagnostic Considerations

Immunohistochemistry (IHC) and cytogenetic analysis are necessary components to the accurate diagnosis and subtyping of DLBCL. There are two "cell of origin" subtypes of DLBCL which have distinct molecular profiles and differing prognoses. The two subtypes are referred to as the germinal center B-cell (GCB) and activated B-cell (ABC) subtypes [10]. The term "non-GCB" is often used to refer to include the ABC cases as well as those which do not clearly fall into the ABC or GCB subtypes. The most accurate method for molecular subtyping of DLBCL is gene expression profiling (GEP). However, until recently, GEP could only be performed on fresh tissue, leading to limited applicability in routine practice. As a result, immunohistochemistry approaches were developed as a surrogate for GEP subtyping. The most commonly used IHC approach for this purpose is referred to as the "Hans algorithm" and uses a combination of IHC staining for CD10, BCL6, and MUM1 [11]. Additional markers including LMO2, GCET1, and FOXP1 have also been proposed in alternative diagnostic algorithms [12]. Recently, techniques have been developed to allow for accurate GEP on archival (formalin fixed) tissue, which may allow for more accurate molecular subtyping of DLBCL in routine practice [13]. Establishing DLBCL subtype is helpful when predicting outcomes to R-CHOP chemotherapy. In patients receiving R-CHOP chemotherapy, GCB subtype has been associated with improved survival when compared to the non-GCB subtype [10]. In addition to "cell of origin" subtyping, most experts now recommend that all new cases of DLBCL be tested for chromosomal rearrangement of the c-*myc* locus. Those cases that are positive for a c-*myc* rearrangement should then be tested for rearrangements of bcl-2

and bcl-6. This approach will accurately classify DLBCL into those without a myc rearrangement, those with a c-myc rearrangement, and those with "double-hit" or "triple-hit" cytogenetics (rearrangement of c-*myc* and/or bcl-2 and/or bcl-6). Myc rearrangement and "double-hit"/"triple-hit" cytogenetics have been associated with inferior outcomes with standard R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone) therapy; as a result more intensive therapy is generally advised in such cases.

Prognostic Factors

The International Prognostic Index (IPI) is an essential part of the initial workup for patients diagnosed with DLBCL. This is a simple prognostic system that relies on several factors that are routinely available on newly diagnosed patients – age, stage, extranodal involvement, LDH, and performance status. To date, several variations of the original IPI have been developed. The two most common prognostic indexes used in clinical practice for DLBCL are the original IPI, including the age-adjusted IPI, and the NCCN-IPI (Tables 32.3a, 32.3b, and 32.3c).

Treatment

First-Line Therapy

Limited Stage (Stage I, II) Disease

The optimal initial treatment for patients with stage I or II DLBCL depends on the presence or absence of bulky disease. Recommended first-line therapy for non-bulky disease, defined as tumor mass diameter <7.5 cm, is R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone) for three cycles with involved-field radiation therapy (IFRT) or R-CHOP for six cycles with or without IFRT [14, 15]. Recommended first-line therapy for bulky disease, defined as tumor mass diameter >7.5 cm, is R-CHOP for six cycles with or without IFRT [16]. Please refer to Table 32.4 for details regarding commonly used first-line combination chemotherapy regimens. In addition, Table 32.5 highlights chemotherapy toxicity and special considerations.

Table 32.3a International prognostic index (IPI)

Prognostic factors	No. of prognostic factors	IPI	5-year OS, %
Age >60 years	0 or 1	Low	73
Serum LDH > normal	2	Low-intermediate	51
ECOG performance status 2–4	3	High-intermediate	43
Stage III or IV	4 or 5	High	26
Extranodal involvement >1 site			

From [64]

Table 32.3b Age-adjusted international prognostic index (age-adjusted IPI)

Prognostic factors (age >60)	No. of prognostic factors	Age-adjusted IPI	5-year OS, %
Stage III or IV	0	Low	56
LDH > normal	1	Low-intermediate	44
ECOG performance status 2–4	2	High-intermediate	37
	3	High	21

From [64]

Table 32.3c NCCN-International Prognostic Index (NCCN-IPI)

Prognostic factors	Risk score	Points	Risk group	5-year OS
Age >40 to ≤60	1	0–1	Low	96
Age >60 to ≤75	2	2–3	Low-intermediate	77
Age ≥75	3	4–5	High-intermediate	56
LDH >1× to ≤3× ULN	1	≥6	High-intermediate	38
LDH >3× ULN	2			
Stage III–IV	1			
Extranodal disease	1			
ECOG performance status 2–4	1			

From [65]

Advanced Stage (Stage III, IV) Disease

Recommended first-line therapy for stage III or IV DLBCL is enrollment in a clinical trial or, off trial, R-CHOP for six cycles [17, 18]. In some patients, radiation therapy to areas of bulky disease may be indicated. The exception to this approach would include cases with myc rearrangement or "double-hit" or "triple-hit" cytogenetics, in which the dose-adjusted EPOCH-R regimen is typically used. Another exception is cases in which there is concurrent systemic and central nervous system involvement at diagnosis. In such cases, high-dose methotrexate and/or high-dose cytarabine is usually incorporated into the regimen. One such approach is to use R-CHOP with high-dose methotrexate given on day 15 of each cycle.

Relapsed and Refractory Disease

In the relapsed and refractory (R/R) setting, high-dose therapy (HDT) with autologous hematopoietic cell transplantation (auto-HCT) is the treatment of choice for fit patients with disease that is sensitive to second-line platinum-based therapy. A number of drug combinations have been used in this setting; however, no single regimen has been shown to be superior to another, and drug selection is often based on toxicity profile and provider preference (Table 32.6). Commonly used drug regimens include R-GDP (rituximab, gemcitabine, dexamethasone, cisplatin), R-DHAP (ritux-

Table 32.4 Commonly used combination chemotherapy regimens: first line

Name of regimen	Drugs	Dosing	
R-CHOP	Rituximab	375 mg/m^2 IV, day 1	
	Cyclophosphamide	750 mg/m^2 IV, day 1	
	Doxorubicin	50 mg/m^2 IV, day 1	
	Vincristine	1.4 mg/m^2 (max dose 2 mg) IV, day 1	
	Prednisone	100 mg PO daily, day 1–5	
DA-EPOCH-R	Rituximab	375 mg/m^2 IV, day 1	
	Etoposide	50 mg/m^2 IV daily, day 1–4	
	Doxorubicin	10 mg/m^2 IV daily, day 1–4	
	Vincristine	0.4 mg/m^2 IV daily, day 1–4	
	Cyclophosphamide	375–750 mg/m^2 IV, day 5	
R-hyperCVAD/ MTX/araC		Cycles 1, 3, 5, 7: rituximab + HyperCVAD	Cycles 2, 4, 6, 8: rituximab plus MTX/araC
	Rituximab	375 mg/m^2 IV, day 1	375 mg/m^2 IV, day 1
	Cyclophosphamide	300 mg/m^2 IV over 3 h q12 h ×6 doses, day 2–4	N/A
	Vincristine	1.4 mg/m^2 (max dose 2 mg) IV, day 5, 12	N/A
	Doxorubicin	16.6 mg/m^2/d IVCI over 72 h, day 5–7	N/A
	Dexamethasone	40 mg IV or PO daily, day 2–5 and 12–15	N/A
	Methotrexate	N/A	200 mg/m^2 IV over 2 h, day 2
		N/A	800 mg/m^2 IVCI over 22h, day 22
	Cytarabine	N/A	3000 mg/m^2 IV over 2 h q12 h ×4 doses, day 3–4
Nordic MCL-2		Cycle 1, 3, 5	Cycle 2, 4, 6
	Rituximab	375 mg/m^2 IV, day 1 (cycle 5 only)	375 mg/m^2 IV, day 1 (cycle 4 and 6 only)
	Cyclophosphamide	1200 mg/m^2 IV, day 1	N/A
	Doxorubicin	75 mg/m^2 IV, day 1	N/A
	Vincristine	2 mg IV, day 1	N/A
	Prednisone	100 mg PO daily, day 1–5	N/A
	Cytarabine	N/A	3 g/m^2 IV over 3 h q12 h ×4 doses, day 1,2
BEAM (auto-HCT conditioning)	BCNU	300 mg/m^2 IV, day 1	
	Etoposide	200 mg/m^2 IV daily, day 1–4	
	Cytarabine	400 mg/m^2 IV daily, day 1–4	
	Melphalan	140 mg/m^2 IV, day 1	

Table 32.5 Chemotherapy toxicity and special considerations

Drug	Toxicity	Special considerations
Rituximab	Infusion reaction	Premedications typically administered and rate of infusion increased gradually to minimize risk of reaction
	Mucocutaneous reactions	Mucocutaneous reaction most common with first dose, incidence decreases with subsequent doses
	Late onset neutropenia	Associated with HBV reactivation and progressive multifocal leukoencephalopathy
Cyclophosphamide	Hemorrhagic cystitis	Coadministration with Mesna and IV fluid hydration used to help minimize risk of bladder toxicity
	Myelosuppression	
Doxorubicin	Cardiotoxicity	MUGA or ECHO required prior to starting treatment, maximum lifetime dose 400 mg/m^2
	Secondary malignancy	Treatment-related AML or MDS have been reported
Vincristine	Neurotoxicity	Symmetric sensory impairment, motor nerve impairment, may be severe if given with pre-existing neurologic conditions
	Constipation	Lethal if administered intrathecally
Etoposide	Hypersensitivity reaction	Premedication often administered to help prevent hypersensitivity
	Myelosuppression	Treatment-related AML or MDS have been reported
Methotrexate	Hepatic	Distributed widely in body tissues and total body water, used with caution in patients with ascites, pleural effusion, or third spacing
	Renal	Urine alkalinization often used to minimize renal toxicity
	Mucositis	
	Myelosuppression	

(continued)

Table 32.5 (continued)

Drug	Toxicity	Special considerations
Cytarabine	Dermatologic	Acral erythema of the hands, hand food syndrome, alopecia
	Neurotoxicity	Acute cerebellar syndrome has been reported, higher risk in patients over 60–65 years old
	Myelosuppression	Myelosuppression greater with continuous infusion when compared to IV bolus
BCNU (carmustine)	Pulmonary	Dose related, can be delayed, significantly higher risk with cumulative dose greater than 1400 mg/m^2
	Myelosuppression	
Melphalan	Secondary malignancy	Treatment-related leukemia has been reported
	Hypersensitivity reaction	Premedications often given to help minimize risk of reaction
	Mucositis	Oral cryotherapy during infusion can decrease risk of mucositis
	Diarrhea	
	Myelosuppression	
Ifosfamide	Hemorrhagic cystitis	Coadministration with Mesna and IV fluid hydration used to help minimize risk of bladder toxicity
	Neurotoxicity	Crosses blood brain barrier and has been associated with encephalopathy
	Myelosuppression	
Carboplatin	Hypersensitivity reaction	Incidence of hypersensitivity seems to increase with number of cycles administered
	Myelosuppression	
Cisplatin	Neurotoxicity	Peripheral neuropathy recovery is typically incomplete
	Ototoxicity	Pretreatment hearing evaluation often considered
	Nephrotoxicity	Aggressive IV hydration used to enhance urinary excretion
Gemcitabine	Myelosuppression	Cases of capillary leak syndrome, posterior reversible encephalopathy syndrome, and hemolytic uremic syndrome have been reported
	Thrombotic microangiopathy	
	Pulmonary	

Table 32.6 Commonly used combination chemotherapy regimens: second line

Name of regimen	Drugs	Dosing
R-ICE	Rituximab	375 mg/m^2 IV, day 1
	Ifosfamide	5000 mg/m^2 IVCI over 24 h, day 2
	Mesna	3300 mg/m^2 IVCI over 24 h, day 2 (with ifosfamide)
		1700 mg/m^2 IVCI over 12 h, day 3 (after ifosfamide)
	Carboplatin	AUC 5; dose = 5 × [25 + Clcr], max 800 mg, day 2
	Etoposide	100 mg/m^2 IV daily, day 1–3
R-DHAP	Rituximab	375 mg/m^2 IV, day 1
	Dexamethasone	40 mg PO daily, day 3–6
	Cytarabine	2 g/m^2 IV over 2 h q12h ×2 doses, day 4
	Cisplatin	25 mg/m^2 IVCI daily, day 3–6
R-GDP	Rituximab	375 mg/m^2 IV, day 1
	Gemcitabine	1000 mg/m^2 IV, day 1,8
	Dexamethasone	40 mg PO daily, day 1–4
	Cisplatin	75 mg/mg IV, day 1

imab, dexamethasone, high-dose cytarabine, cisplatin), and R-ICE (rituximab, ifosfamide, carboplatin, etoposide). R-ICE is the most commonly used regimen in the USA. All patients under age 75 years with relapsed and refractory DLBCL who demonstrate chemosensitive disease should be considered for HDT with auto-HCT with or without radiation therapy (RT). Other considerations in this setting could include a clinical trial or allogeneic hematopoietic cell transplantation (allo-HCT). Patients who are not candidates for HDT with auto-HCT should be evaluated for clinical trials or palliative therapies which include bendamustine, gemcitabine, oral regimens such as CEPP (cyclophosphamide, etoposide, procarbazine, prednisone), or other alternative combination chemotherapy regimens [8]. Newer targeted or immunotherapy agents such as lenalidomide, ibrutinib, brentuximab, nivolumab, or pembrolizumab may have activity in some cases but in large part are still considered investigational approaches not currently FDA approved for this indication, with the exception of pembrolizumab which was approved in June 2018 for relapsed primary mediastinal DLBCL. Chimeric antigen receptor, or CAR-T cell therapy, has recently been approved for relapsed/refractory DLBCL. Approximately 50–60% will achieve complete remission with CAR-T therapy. Of these, roughly half will be durable remissions at 6–12 months. Whether these remissions truly represent cures will require longer follow-up. Prospective randomized trials comparing CAR-T cell therapy and autologous HCT are on-going.

Double-Hit Lymphoma

The term "double-hit" lymphoma (DHL) refers to de novo DLBCL with translocation of c-MYC as well as a

Table 32.7a Mantle cell lymphoma international prognostic index (simplified MIPI)

Points	Age (years)	ECOG PS	LDH:ULN ratio	WBC(10^9/L)	Total score	Risk group	5-year OS, %
0	<50	0–1	<0.67	<6.7	0–3 points	Low	60
1	50–59		0.67–0.99	6.7–9.9	4–5 points	Intermediate	35
2	60–69	2–4	1.00–1.49	10.0–14.9	6–12 points	High	20
3	≥70		≥1.50	≥15.0			

From [24]

translocation of BCL2 or BCL6, as detected by cytogenetic analysis, usually fluorescent in situ hybridization (FISH). If all three rearrangements are present, the term "triple hit" has been used. The term "double-expressor" lymphoma (DEL) refers to DLBCL cases in which high levels of c-myc and bcl-2 protein are detected by immunohistochemistry. True (cytogenetic) double-hit lymphoma occurs in approximately 5% of newly diagnosed DLBCL and is associated with a worse progression-free survival and overall survival when compared to other DLBCLs. Double-expressor DLBCL is seen in about 30% of newly diagnosed DLBCL and also has a less favorable prognosis. No standard of care has been established for treatment of DHL or DEL. The use of more intensive induction regimens such as DA-EPOCH-R, R-hyperCVAD, or CODOX-M/IVAC leads to improved outcomes in DHL and is therefore commonly utilized for patients under age 70–75 [19, 20]. It is unclear whether specific regimens improve outcomes for DEL. Enrollment in a clinical trial is strongly recommended when available for patients with DHL and DEL.

Mantle Cell Lymphoma (MCL)

Diagnostic Considerations

MCL represents approximately 6% of all newly diagnosed NHL in the USA. [21]. Histologic examination, cytogenetic analysis, and immunohistochemistry are used to confirm the diagnosis. Typically, MCL will be positive for CD5 and CD20, with overexpression of cyclin D1, overexpression of SOX11, and a t(11;14) translocation [22]. While MCL typically follows an aggressive trajectory, there is a minority that will follow a more indolent course and for whom immediate treatment may be safely deferred [23].

Prognostic Factors

Since the advent of the original IPI, various disease-specific prognostic models have been developed for different NHL subtypes. For example, the mantle cell lymphoma international prognostic index ("MIPI score") was developed based on analysis of patients from three European clinical trials, including a total of 455 patients with advanced stage MCL. Prognostic factors include age, LDH, WBC count, and ECOG performance status. Prognostic factors are used to calculate a score and stratify patients into low-, intermediate-, and high-risk groups. Five-year overall survival (OS) was demonstrated to be 60%, 35%, and 20% for the low-, intermediate-, and high-risk groups, respectively [24] (Tables 32.7a and 32.7b). Since its original description, this prognostic tool has been validated in several other independent datasets, including patients treated with intensive or non-intensive first-line strategies. In addition an enhanced version of the MIPI score (the "combined MIPI" or "MIPI-c") incorporates the proliferation rate as well [25]. Various online calculators and apps have been developed to simplify clinical integration of these prognostic indices.

Table 32.7b Mantle cell lymphoma international prognostic index (MIPI score)

MIPI score	Risk group	% of patients	Median OS
<5.7	Low	44%	Not reached
5.7 to <6.2	Intermediate	35%	51 months
≥6.2	High	21%	29 months

From [24]
MIPI score is calculated based on the following equation: [0.03535 × age (years)] + 0.6978 (if ECOG >1) + [1.367 × log10(LDH/ULN)] + [0.9393 × log10(white cells per uL blood)]

Treatment

Frontline

Initial treatment for MCL is guided by patient age, fitness, and treatment goals. For younger, fit patients the treatment associated with the longest first remission is a rituximab and high-dose cytarabine-based induction regimen, followed by consolidation with auto-HCT. Three such regimens commonly used for induction include R-CHOP alternating with R-DHAP [26], the Nordic MCL-2 regimen (dose-intensified induction immunochemotherapy with rituximab plus cyclophosphamide, vincristine, doxorubicin, prednisone [maxi-CHOP] alternating with rituximab + high-dose cytarabine) [27] or R-DHAP × 4 cycles [28]. These induction regimens are then typically consolidated with high-dose therapy and auto-HCT. The R-hyperCVAD/R-MTX-araC regimen is another intensive induction used in some centers [29]. This regimen not typically followed by auto-HCT consolidation. However, although some patients will achieve long-term (>10 year) remission with this treatment, the regimen has significant toxicity, with a treatment dropout rate ranging from 29% to 63% [29–31]. Overall, these intensive induction

strategies are associated with long first remission (median first remission in the 6–10-year range), but the trade-off is treatment-related mortality in the 1–8% range and a small but real risk of secondary malignancies such as myelodysplastic syndrome or acute myeloid leukemia. In addition, while it is clear that median progression-free survival is superior, it remains an area of debate as to whether intensive first-line therapy truly prolongs overall survival, particularly in the modern era in which multiple agents are available for patients with relapsed and refractory MCL. Current areas of research involve incorporating novel agents with activity in the relapsed/refractory setting, into frontline regimens, and development of risk-adapted first-line therapy. One example of the latter is the recently activated ECOG-ACRIN 4151 trial, in which patients who achieve a minimal residual disease (MRD)-negative CR are then randomized to auto-HCT followed by 3 years of maintenance rituximab, versus 3 years of maintenance rituximab (and deferral of auto-HCT) [32].

For patients who are unfit for aggressive therapy, various chemotherapy backbones have been studied. There is no standard therapy in this setting, but common regimens include bendamustine + rituximab [33], VR-CAP (bortezomib, rituximab, cyclophosphamide, doxorubicin, and prednisone) [34] and R-CHOP followed by maintenance rituximab [35]. For patients who respond to first-line therapy, but who are not candidates for auto-HCT, rituximab maintenance (generally one dose every 2 months for 2 years) is commonly employed although the benefit of maintenance rituximab in this setting has only formally been proven following R-CHOP induction.

Relapsed/Refractory MCL

Various salvage treatment regimens have been evaluated in relapsed and refractory MCL. There is no widely accepted standard of care, and drug selection is typically based on prior therapy, patient comorbidities, concomitant medications, and performance status. Possible regimens in this setting include ibrutinib, lenalidomide, bortezomib, or bendamustine. Each of these agents can be combined with rituximab as well. Other multi-agent combination chemotherapy regimens can be used as well, such as those described above for R/R DLBCL. Ibrutinib is an oral Bruton tyrosine kinase inhibitor with single-agent response rates in the 65–70% range in relapsed/refractory MCL. Lenalidomide is an immunomodulatory and antiangiogenic agent with antineoplastic activity. In one phase II trial studying lenalidomide in relapsed and refractory MCL, the overall response rate for lenalidomide was 28% with 7% complete response and a median duration of response of 16.6 months (95% CI 7.7–26.7 months) [36]. When combined with rituximab, a higher response rate (57%) was seen [37]. Bortezomib is a proteasome inhibitor which produces responses in about 30% of MCL relapsed/refractory MCL [38]. Ultimately, in patients who demonstrate a clinical response to salvage therapy, hematopoietic cell transplantation (either autologous or allogeneic) should be considered. Allo-HCT has more initial risk of treatment-related mortality in the first 1–2 years but is potentially curative in R/R MCL [39], whereas auto-HCT in general only provides about 2–3 years of disease control in R/R MCL [40, 41].

Burkitt Lymphoma

Diagnostic Considerations

Burkitt lymphoma (BL) can be classified into endemic, sporadic, and immunodeficiency-associated clinical variants. Endemic BL is typically associated with Epstein-Barr virus (EBV) and is one of the most common causes childhood malignancy in areas of Africa. In the USA, the sporadic and immunodeficiency-associated variants are most commonly encountered. Sporadic BL is also associated with EBV in up to 30% of cases and comprises approximately 2% of all lymphomas in the USA and Europe. Immunodeficiency-associated BL has been described in the posttransplant setting, in patients with HIV and in congenital immunodeficiency states.

Diagnosis for BL depends on histology, immunohistochemistry, and cytogenetic analysis. A typical diagnostic profile for BL includes monomorphic, medium-sized cells with round nuclei and deeply basophilic cytoplasm (usually with many vacuoles present), with low-power histology often described as a “starry sky” pattern. Immunophenotypically, BL is usually positive for CD10, CD19, and CD20. TdT is negative and bcl2 should also be negative. Proliferation rate (as measured by immunohistochemical staining with Ki-67 or MIB-1) is 90% or higher, often approaching 100%. For a true Burkitt lymphoma, a translocation involving the c-myc locus on chromosome 8 should be present. The c-myc translocation can involve different partner genes, specifically, the immunoglobulin heavy chain or light chain loci (kappa or lambda). As a result the translocations seen are either t(8;14), t(2;8), or t(8;22), with t(8;14) being most common [42].

Prognostic Factors

Various prognostic factors have been evaluated in BL. One prognostic model developed for BL calculates a risk score by assigning 1 point for age 40–59 years or Black race, 2 points for age equal to or greater than 60 years or stage III/IV, and 4 points for age equal to or greater than 80 years. Risk stratification into low risk (0–1 points), low-intermediate risk (2 points), high-intermediate risk (3 points), high risk (4 points) corresponded with 5-year relative survival of 71%, 55%, 41%, and 28%, respectively [43].

The CODOX-M/IVAC regimen (cyclophosphamide, doxorubicin, vincristine with intrathecal methotrexate, and

cytarabine followed by high-dose systemic methotrexate ± rituximab), also known as the Magrath regimen [44], was evaluated in a phase II study where low-risk (LR) patients were treated with three cycles of modified CODOX-M, while high-risk (HR) patients received treatment with four cycles of alternating modified CODOX-M and IVAC chemotherapy. LR was defined as having all of the following features: normal LDH level, WHO performance status of 0 or 1, Ann Arbor stage I–II, and no tumor mass ≥10 cm. All remaining patients were considered HR. Overall, 2-year event-free survival (EFS) was 64.6% (95% CI 50.4–78.9%) and 2-year overall survival (OS) was 72.8% (95% CI 59.4–86.3%). However for LR patients, the 2-year EFS was 83.3% and OS was 81.5%, whereas for HR patients, 2-year EFS was 59.5% and OS was 69.9% [45].

The St. Jude's staging system is another schema used to assess risk in BL. Some clinicians prefer this system because of its ability to better characterize the extent of extranodal disease. It should be noted, however, that this staging system was developed during the era of debulking surgery, which is no longer standard of care [46].

Treatment

First-Line

Various combination chemotherapy regimens have been studied in BL. These regimens, which are intensive, multiagent regimens with aggressive CNS prophylaxis, are the standard of care and are clearly superior to less intensive regimens such as R-CHOP. As a result, other than possibly in elderly or frail patients, R-CHOP is considered an inadequate induction regimen for BL. Patients with BL are at very high risk for tumor lysis syndrome (TLS), and treatment should include aggressive measures for TLS prophylaxis and treatment. In addition, due to the intensive nature of the regimens employed for BL, aggressive supportive care is required, including blood and platelet transfusions, as well as prophylaxis and treatment for neutropenic fever.

Commonly used regimens for newly diagnosed younger and fit BL patients include the Magrath (CODOX-M/IVAC) regimen [45], DA-EPOCH-R [47], and R-Hyper-CVAD [48]. When delivered properly, cure rates in the 60–70% range can be achieved. Individual chemotherapy selection is often based on patient age, comorbidities, and provider preference [8].

Relapsed/Refractory

There is no established second-line therapy for BL. Considerations include DA-EPOCH-R, R-ICE (rituximab, ifosfamide, carboplatin, etoposide), R-IVAC (rituximab, ifosfamide, cytarabine, etoposide), R-GDP (rituximab, gemcitabine, dexamethasone, cisplatin), and high-dose cytarabine plus rituximab. Selection often takes into consideration previous regimens and patient comorbidities [8]. Long-term remission is rarely seen in patients who relapse after an intensive first-line therapy; however, rare patients who undergo auto- or allo-HCT in second remission may be cured, particularly if they go into transplant in a complete remission state and if the first-line therapy was non-intensive [49].

Transformed Lymphoma

Introduction

Histologic transformation (HT) of indolent lymphoma to a more aggressive subtype is relatively common. Follicular lymphoma (FL) is one of the most common indolent lymphomas associated with HT; however, HT can occur with various other indolent subtypes such as small lymphocytic lymphoma/chronic lymphocytic leukemia (SLL/CLL), lymphoplasmacytic lymphoma/Waldenstrom macroglobulinemia (LPL/WM), and marginal zone lymphoma (MZL).

Epidemiology

For FL, the incidence of HT at 10 years has been reported to range from 15% to 31%. In lymphoma subtypes other than FL, the 10-year risk of HT ranges from 7% to 16%. There is some suggestion that the incidence of HT may be lower in the rituximab era [50]. It is thought that, on average, patients with indolent lymphomas have an approximately 1–2% risk per year of HT. That risk appears to decrease after patients are 10 or more years since diagnosis.

Clinical Presentation

Clinicians should suspect HT in patients with a previously diagnosed indolent lymphoma who subsequently develop rapidly progressive lymphadenopathy, disease progression into uncommon extranodal sites, an abrupt change in performance status, new systemic symptoms (fever, night sweats, or weight loss), or new laboratory abnormalities including elevated lactate dehydrogenase (LDH), elevated uric acid, or hypercalcemia.

Diagnosis

Once HT is suspected, an excisional biopsy of involved tissue, most commonly a lymph node, should be pursued. PET/CT can be useful to identify the most FDG-avid disease site amenable to excisional biopsy. Diagnosis ultimately relies on histopathologic evaluation. In most cases, indolent lymphoma transforms to DLBCL (including "double-hit" lymphoma); however, Burkitt lymphoma and other aggressive non-Hodgkin lymphoma subtypes such as lymphoblastic lymphoma can be seen.

Treatment

For patients who have received little or no prior therapy, initial treatment mirrors that of the aggressive histology which

the lymphoma has transformed into. For example, for a transformation from FL to DLBCL, R-CHOP would therefore be recommended as first line. It is important in such cases, however, to rule out a "double-hit" lymphoma. For patients who have received prior therapy, chemoimmunotherapy with R-CHOP is still recommended if an anthracycline regimen was not previously given. For those who have had prior R-CHOP, platinum-based regimens such as those discussed for relapsed/refractory DLBCL are recommended. For those with chemosensitive disease, consolidation with HDT followed by auto-HCT (and, less commonly, allo-HCT) is often recommended and can result in long-term remission [51]. For relapsed or refractory disease, treatment options mirror that for R/R de novo DLBCL.

Aggressive Mature Peripheral T-Cell Lymphomas

Peripheral T-Cell Lymphoma, Not Otherwise Specified

Diagnostic Considerations

Peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS) represents the most common PTCL subtype, accounting for approximately 25–30% of all PTCLs [52]. PTCL-NOS is essentially a diagnosis of exclusion, in which pathologic evaluation shows a mature T-cell NHL, without meeting criteria for a specific PTCL subtype. In many cases, histology demonstrates atypical large lymphoid cells, with immunohistochemistry or flow cytometry then used to confirm T-cell origin. Immunophenotype for PTCL-NOS is variable; however, expression of one or more T-cell antigens including CD2, CD3, CD5, or CD7 is essential for the diagnosis. B-cell markers are usually absent. CD30 is also included in upfront diagnostic testing to guide utility of treatment with brentuximab vedotin. Demonstration of a clonal T-cell population using PCR or flow cytometry can be useful in cases in which the diagnosis is unclear or in cases for which there is limited diagnostic material.

Prognostic Factors

Various prognostic models have been evaluated for patients with PTCL-NOS. The most commonly used models include the original IPI score and the prognostic index for PTCL-NOS (the "PIT score"). The PIT score was developed based on a retrospective, multicenter study of 385 patients with PTCL-NOS. Four criteria were found to be prognostic, including age >60 years, increased serum LDH, ECOG PS of 2 or greater, and bone marrow involvement. Patients were stratified into four risk groups defined by having zero, one, two, or three to four prognostic factors. The associated 5-year overall survival was 63%, 53%, 33%, and 18%, respectively [53] (Table 32.8).

Table 32.8 Prognostic index for peripheral T-cell lymphoma, unspecified (PIT)

Prognostic factor	No of prognostic factors	Risk group	5-year OS (%)
Age >60 years	0	1	63
Elevated LDH	1	2	53
ECOG PS >1	2	3	33
Bone marrow involvement	3–4	4	18

From [53]

Anaplastic Large Cell Lymphoma

Diagnostic Considerations

Based on the current WHO classification, there are two distinct subtypes of systemic anaplastic large cell lymphoma (ALCL), namely, anaplastic lymphoma kinase (ALK)-positive ALCL and ALK-negative ALCL. ALK-positive ALCL often presents in children and young adults and is characterized by overexpression of the ALK-1 protein. In 40–60% of patients, this is a result of a translocation t(2;5) [54]. Conversely, ALK-negative ALCL typically presents in older patients, with median age at presentation in the late 50s, and ALK expression is absent. The immunophenotype for ALCL is typically CD2+, CD4+, CD5+, and CD7+. CD30 is a hallmark of ALCL and is important to document for determining treatment options, particularly in the relapsed or refractory setting.

Prognostic Factors

In newly diagnosed patients, ALK-positive ALCL is associated with improved prognosis when compared to ALK-negative ALCL, although this improved prognosis tends to decrease with increased age and higher prognostic risk scores. According to the International T-cell Lymphoma Project, in patients with ALK-positive ALCL, as compared to ALK-negative ALCL, receiving anthracycline-containing regimens, the 5-year failure-free survival (FFS) was 60% vs 36%, $p = 0.015$, and the OS was 70% vs 49%, $p = 0.016$, respectively [55]. Approximately 30% of ALK-negative ALCL have chromosomal rearrangement of DUSP22. DUSP22 rearranged ALK-negative ALCL have favorable outcomes similar to ALK-positive ALCL [56].

Angioimmunoblastic T-Cell Lymphoma

Diagnostic Considerations

Angioimmunoblastic T-cell lymphoma (AITL) is the second most common PTCL accounting for approximately 15–20% of cases. The median age at diagnosis is 65 years and most patients present with advanced disease. Clinical presentation can be quite dramatic with severe B symptoms, pleural effu-

sions, ascites, non-specific skin manifestations, and dysproteinemia. Many of the presenting signs and symptoms may mimic other infectious or inflammatory conditions making the diagnosis difficult to establish. Histologically, affected lymph nodes are replaced by a diffuse, polymorphous cell population associated with branching high endothelial venules. Many cases also demonstrate B-cell populations positive for EBV. Immunophenotyping typically shows expression of T-cell antigens (CD2, CD3, and CD5) and T-helper cell antigens (CD4, CD10, BCL6, CXCL13, and PD-1) [57].

Prognostic Factors

In clinical practice the original IPI. In one prospective study evaluating 157 patients with AITL receiving CHOP chemotherapy, the 2-year, 5-year, and 7-year overall survival was 51%, 33%, and 29%, respectively [58].

Treatment of Mature Peripheral T-Cell Lymphomas

First Line

For patients with stage I–II ALK-positive ALCL, combination chemotherapy with CHOP or CHOEP with or without IFRT is recommended. In most cases, six cycles of chemotherapy are recommended when IFRT is not used, and three to four cycles of chemotherapy can be considered when IFRT is used. Combination chemotherapy with CHOP or CHOEP for six cycles without IFRT is recommended for stage III–IV ALK-positive ALCL. For patients with ALK-negative ALCL, PTCL-NOS, and AITL, induction with CHOEP (or CHOP if over age 60) is commonly used, with consideration given to consolidation with auto-HCT in first remission. There remains some debate over the true benefit of auto-HCT in first CR for these patients, but in some retrospective studies, the long-term PFS appears somewhat improved for those undergoing auto-HCT in first CR. Allo-HCT is typically not recommended in first CR, although is a consideration for patients with AITL, as registry data indicates favorable long-term outcomes following allo-HCT with AITL appearing to be a lymphoma subtype that is particularly responsive to the graft-versus-lymphoma effect [59]. However, a clinical trial remains the preferred option in this setting when available.

Relapsed and Refractory Disease

Enrollment in a clinical trial is the preferred treatment option for relapsed and refractory PTCL. In the absence of a clinical trial, treatment is typically guided by whether transplantation is a possibility. If a patient is a transplant candidate, second-line therapy is administered, and if a response is observed, auto-HCT or allo-HCT should be considered. Conversely, if a patient is not a transplant candidate, second-line systemic therapy without transplant would be recommended. A number of second-line therapies have been studied in this setting, but there is a paucity of data to suggest one agent over another. Standard platinum-based lymphoma salvage regimens (as discussed previously) can be considered for younger transplant-eligible patients. In addition, there are now several drugs with FDA approval and single-agent activity in mature PTCLs, including belinostat, brentuximab vedotin (the immunoconjugate anti-CD30 targeted therapy), pralatrexate, and romidepsin [60]. For relapsed or refractory ALCL, brentuximab is highly active and should be considered early on [61].

Special Considerations (for All Aggressive NHLs)

CNS Treatment and Prophylaxis

In patients who present with CNS parenchymal involvement, systemic methotrexate at a dose between 3 and 8 g/m^2, often repeated every 2 weeks, is recommended. Whole brain or craniospinal radiation can also be considered. For CNS leptomeningeal disease without parenchymal involvement, either systemic methotrexate or intrathecal chemotherapy with methotrexate or cytarabine are options. For patients without evidence of CNS disease at initial presentation, CNS prophylaxis may be given for those felt to be at high risk for CNS relapse. There are various risk assessment tools for this purpose; one very useful and robust tool is the CNS-IPI score which incorporates various independent risk factors including bone marrow, adrenal, or kidney involvement, presence of B symptoms, and low serum albumin concentration [62]. The optimal regimen for CNS prophylaxis remains unclear. Both systemic methotrexate and intrathecal chemotherapy are utilized depending on the clinical situation. For patients felt to be at high risk for CNS relapse, high-dose systemic methotrexate is preferred since it will penetrate brain parenchyma as well as CSF, whereas intrathecal chemotherapy will only prophylax the CSF. In addition, injection of chemotherapy via lumbar puncture may not always result in consistent distribution throughout the CSF, for example, if CSF flow is impeded or blocked due to gross tumor involvement.

Investigational/Emerging Therapies

In recent years, several very exciting new treatmentoptions have emerged, including signal transduction inhibitors, new monoclonal antibodies, antibody-drug conjugates, and immune checkpoint inhibitors. All of these approaches have been (or are still) under evaluation in aggressive NHLs and

have shown early promising results certain settings, but to date have not become FDA-approved or standard-of-care therapies [63]. Exceptions to this would be the use of the antibody-drug conjugate brentuximab vedotin for ALCL [61]. It is expected that in the coming years some of these agents will become established as effective components of therapy for aggressive NHLs.

Conclusions

The distinction between indolent and more aggressive forms of NHL is useful as the two groups have variable clinical presentation, chemosensitivity, potential curability, and overall survival, each of which affects the clinical approach to prognostication and treatment. Accurate diagnosis is of utmost importance in determining an appropriate treatment plan. In general, the treatment options and clinical outcomes for aggressive NHL continue to improve, although for some subtypes, the outcomes remain poor even with modern therapy. In recent years there has been an expanding knowledge of the pathobiology underlying aggressive NHLs and a growing hope for improved patient outcomes as new and novel treatments are developed.

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Clinical Evaluation and Management of Chronic Lymphocytic Leukemia

33

Nitin Jain and Susan O'Brien

Introduction

Chronic lymphocytic leukemia (CLL) is characterized by clonal expansion of CD5-positive B cells. The last few years have seen significant advances in the field of CLL, both in the understanding of the disease biology and in the treatment options [1].

Epidemiology

CLL is the most common leukemia in the United States, with an estimated incidence of 20,110 new CLL cases and 4660 deaths in 2017 [2]. The number of new cases of CLL was 4.7 per 100,000 people per year. Based on the SEER 18 database (year 2010–2014), the median age at diagnosis is 70 years. The incidence of CLL increases with age, and CLL is most frequently diagnosed among people aged 65–74 years. CLL is very uncommon in less than 45 years' age group. The 5-year survival (SEER 18) for patients with CLL is 83.2%. CLL is more common in men than women. Caucasians have a higher risk of developing CLL than other races. The disease is very uncommon in Asians [3]. The risk of CLL is not increased in Asians settled in the Western countries, indicative of the important role of genetic factors [4]. In population studies, a higher risk of CLL is seen in individuals living on farms or those exposed to Agent Orange, but not in those exposed to ionizing radiation [5, 6]. Up to 10% of patients with CLL have a family history of CLL or a related lymphoproliferative disorder, and several single nucleotide polymorphisms (SNPs) have been associated with an increased risk of CLL [7].

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Biology

Morphologically CLL cells resemble small mature lymphocytes. CLL cells are monoclonal and express CD5, along with B cell markers such as CD19, CD20, and CD23. Surface immunoglobulin and FMC7 are typically weak or negative in CLL cells.

Assessment for somatic hypermutation of immunoglobulin heavy chain variable (*IGHV*) gene defines two subsets of CLL: mutated *IGHV* CLL >2% deviation from germline sequence) and unmutated *IGHV* CLL (less than or equal to 2% deviation from germline sequence). Mutated *IGHV* CLL appear to arise from postgerminal B cells, while the unmutated *IGHV* CLL appear to arise from naive B cells [8, 9]. Unmutated *IGHV* is associated with inferior clinical outcomes in the context of chemoimmunotherapy [10, 11]. Zeta-associated protein 70 (ZAP-70) and CD38, originally developed as surrogates for *IGHV* mutation status, are associated with inferior outcomes [8, 12, 13]. The low mitotic rate of CLL generally hampers conventional cytogenetic assessment. B cell mitogens may be used to enhance the yield. Complex karyotype is recognized as a high-risk factor in the context of targeted therapies [14]. Fluorescence in situ hybridization (FISH) performed on interphase cells is now a standard evaluation for help in defining prognosis and for treatment selection. In a seminal paper by Dohner and colleagues, deletion 13q was reported as the most common genetic abnormality by FISH (55%), followed by deletion 11q (18%), trisomy 12 (16%), and deletion 17p (7%) [15]. The median survival for the patients correlated with the FISH categories [15]. Clonal evolution leads to acquisition of new genetic abnormalities that may have therapeutic implications; therefore, FISH assessment should be repeated at the time of disease relapse before the start of a new therapy. DNA sequencing has identified several recurrently mutated genes (including but not limited to *TP53*, *NOTCH1*, *SF3B1*, *BIRC3*, *MYD88*, *XPO1*, *POT1*, *ATM)* in patients with CLL, many of which have prognostic significance [16, 17]. *TP53* mutations

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are typically associated with deletion 17p, and *ATM* and *SF3B1* mutations are typically associated with deletion 11q.

Clinical Features

The diagnosis of CLL is often incidental with lymphocytosis noted on routine blood counts. Some patients are diagnosed to have CLL when they have imaging studies performed for unrelated medical issues. Some patients may present with palpable adenopathy. B symptoms (fever, weight loss, night sweats) are generally uncommon at the time of initial diagnosis. Exaggerated skin reaction to insect bites is frequent in CLL. Some patients can present with anemia and/or thrombocytopenia and consequent symptoms such as fatigue, bleeding, etc. Physical examination may reveal lymphadenopathy, splenomegaly, and/or hepatomegaly.

Laboratory Features and Diagnosis

The diagnosis of CLL requires the presence of greater than or equal to 5×10^9/L clonal B-lymphocytes in the peripheral blood. The clonal B cell population, detected by flow cytometry, is generally positive for light chain restriction (either kappa or lambda); CD5, CD19, CD23, and CD79b; surface immunoglobulin expression; and low levels of CD20 [18]. The CLL cells resemble mature lymphocytes with dense chromatin and scant cytoplasm. A bone marrow biopsy is typically not required for the diagnosis of CLL. The bone marrow is generally hypercellular for age, and infiltration pattern can be nodular, interstitial, or diffuse. Anemia or thrombocytopenia may be present, either from the CLL infiltration of the marrow or from immune destruction.

Up to 5% of otherwise normal individuals over the age 40 years may harbor a population of monoclonal B cells, which when $<5 \times 10^9$/L and associated with no symptoms or cytopenias, is termed monoclonal B lymphocytosis (MBL) [19]. It is estimated that the rate of progression from MBL to CLL is 1–2% per year. Patients with a clonal B cell count of $<5 \times 10^9$/L but with lymphadenopathy or organomegaly are diagnosed with small lymphocytic lymphoma (SLL).

Prognosis

The Rai classification defined five stages from 0 to IV, based on the presence of lymphocytosis, lymphadenopathy, organomegaly, and cytopenias. The Binet classification categorized patients into different prognostic groups in a similar fashion. FISH, *IGHV* mutation status, serum β2-M, ZAP-70 expression, and CD38 expression are well-established prognostic factors in CLL. Several other prognostic markers such as somatic mutations in *TP53*, *NOTCH1*, *SF3B1*, and *BIRC3* have prognostic implications. Recently the CLL international prognostic index (CLL-IPI) was formulated based on age, serum β2-M, Rai stage, presence of *TP53* aberration, and *IGHV* mutational status [20–22].

Treatment

Indications for Treatment

Most patients with CLL do not need treatment at the time of diagnosis and are generally placed on active observation that can last for several years. The NCI-IWCLL criteria for the treatment of CLL are listed in Table 33.1.

In the era of chemoimmunotherapy, early treatment of patients with CLL not meeting treatment indications was not shown to be beneficial. However, in the era of targeted therapies, this question is being investigated again, especially for patients with high-risk genetic features.

Initial Treatment

Treatment options for patients with CLL have changed significantly in the last few years. Chemoimmunotherapy (CIT) has been the standard first-line treatment for patients with CLL; however, this is undergoing evolution with the introduction of targeted therapies [1].

Purine Analogues and Alkylating Agents

Alkylating agents such as chlorambucil or cyclophosphamide served as the main treatment options for patients with CLL for several decades. In late 1980s, the activity of fludarabine, a purine analog, was noted in patients with CLL [23, 24]. Subsequently, randomized trials compared the efficacy of fludarabine + cyclophosphamide (FC) versus fludarabine alone in previously untreated CLL and reported superior progression-free survival (PFS) for the FC arm [25–27]. Bendamustine is an alkylating agent that has structural similarities to a purine analog. A phase III trial showed a higher CR rate and longer PFS with bendamustine as compared to that seen with chlorambucil in patients with previously untreated CLL [28].

Table 33.1 Treatment indications in CLL

Progressive bone marrow failure: anemia and/or thrombocytopenia
Progressive lymphadenopathy
Progressive hepatomegaly or splenomegaly
Progressive lymphocytosis (doubling time <6 months)
Progressive constitutional symptoms
Steroid-refractory autoimmune hemolytic anemia and/or immune thrombocytopenia

Monoclonal Antibodies

Rituximab is a chimeric antibody that targets the CD20 antigen. It causes cell lysis by various mechanisms such as antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and direct apoptosis. Single-agent rituximab has limited activity in patients with CLL. Ofatumumab, a fully human IgG1 mAb, binds to a different epitope of CD20 than rituximab and produces more CDC as compared to that seen with rituximab [29]. In a trial of ofatumumab monotherapy (weekly for 8 weeks, followed by 4 monthly infusions) in patients with fludarabine- and alemtuzumab-refractory CLL, or fludarabine-refractory CLL with bulky lymph nodes, an overall response rate (ORR) of around 45% and median PFS of 5 months were noted [30]. Obinutuzumab is a humanized type II CD20 mAb with a glycoengineered Fc domain that leads to enhanced ADCC as compared to that seen with rituximab. CD20 monoclonal antibodies are being studied in combination with chemotherapy and with targeted therapies. Alemtuzumab targets the CD52 antigen that is routinely expressed on CLL cells. Alemtuzumab has been studied both as a single agent and in combination with chemotherapy. However, with the advent of targeted therapies as well as the serious infectious complications seen with alemtuzumab (such as CMV reactivation), alemtuzumab now has a very limited role in the treatment of CLL.

Chemoimmunotherapy

After the discovery of the clinical activity of CD20 monoclonal antibodies in CLL, subsequent trials investigated combinations of CD20 monoclonal antibodies with chemotherapy. The group at MD Anderson Cancer Center developed a chemoimmunotherapy (CIT) regimen called FCR (fludarabine, cyclophosphamide, rituximab) [31]. Fludarabine was given for 25 mg/m^2 per day for 3 days, the cyclophosphamide dose was 250 mg/m^2 per day for 3 days, and rituximab 375–500 mg/m^2 was given once per each cycle. A total of six cycles were administered. The ORR was 95% (CR 72%, nodular partial remission (PR) 10%, PR 13%). In a recent paper reporting long-term outcomes of patients treated with FCR (median follow-up of >12 year for the surviving patients), the median PFS was 6.4 years [11]. The German CLL Study Group (GCLLSG) conducted a randomized control trial comparing FCR with FC (CLL8 trial) as initial therapy of CLL [10]. A total of 817 patients were randomized; median age was 61 years (range, 30–81). The FCR regimen led to a higher CR rate (44% vs. 22%, $p < 0{\cdot}0001$), as well as superior PFS (median 52 months vs. 33 months, $p < 0{\cdot}0001$), and OS (3-year 87% vs. 83%, p 0.012).

Bendamustine in combination with rituximab (BR regimen) has been evaluated for firstline treatment for patients with CLL [32]. A total of 117 patients were treated with an ORR of 88%, CR rate 23%, and median PFS of 34 months. The GCLLSG CLL10 trial compared FCR with BR in a randomized fashion in patients with previously untreated CLL (CIRS <6 and creatinine clearance greater than or equal to 70 ml/min) [33]. Patients with del(17p) were excluded. A total of 561 patients were included in the intention-to-treat analyses (282 patients in the FCR arm; 279 in the BR arm). The median PFS was 41·7 months with BR vs. 55.2 months with FCR (HR 1·643, $p = 0.0003$). There was no difference in OS (at 3 years, 92% BR vs. 91% FCR). The median number of chemotherapy cycles administered in both arms was 6. A total of 29% patients in the FCR arm, and 19% in BR received less than the planned 6 cycles ($p = 0.005$). Dose reduction (for any of the treatment drugs) occurred in 52% of patients in both the FCR and BR arms. Myelosuppression and infections were more frequently observed with the FCR regimen. Among patients aged >65 years, a higher proportion of patients in the FCR arm (43%) were unable to complete the planned six cycles (BR arm 24%) ($p = 0.013$). There was no difference in the median PFS in the FCR and BR arms among patients >65 years. The CLL10 trial established the superiority of FCR over BR as the CIT regimen of choice for patients <65 years of age. BR is preferred over FCR for patients with moderate renal dysfunction such as those with GFR between 30 and 70 ml/min, as FCR therapy is poorly tolerated in patients with GFR <70 ml/min, likely due to the need for renal excretion of fludarabine.

For patients with CLL with significant comorbidities, and in those >65 years of age who may not be able to tolerate CIT regimens such as FCR/BR, chlorambucil has been investigated in combination with a CD20 mAb. In a phase III trial, patients with CLL with coexisting conditions (CIRS score >6 and/or creatinine clearance 30–69 mL/min) were randomized to receive chlorambucil monotherapy, chlorambucil plus rituximab, or chlorambucil plus obinutuzumab ($N = 781$) [34, 35]. Both the antibody arms produced longer PFS and OS than did chlorambucil alone. The median PFS was significantly better in the obinutuzumab-chlorambucil arm compared to that in the rituximab-chlorambucil arm (median PFS 29.2 vs. 15.4 months, $p < 0.001$) [34, 35]. This trial led to the FDA approval of the combination of obinutuzumab and chlorambucil in patients with previously untreated CLL. In another trial, patients who were deemed ineligible for FCR-based regimens were randomized to chlorambucil monotherapy vs. chlorambucil + ofatumumab (COMPLEMENT-1 trial) [36]. The median PFS was superior for the antibody arm (22.4 months vs. 13.1 months, $p < 0.001$) [36].

Stratification by *IGHV* mutation status and long-term outcomes with CIT: In the last few years, several reports have indicated highly favorable long-term outcomes after firstline FCR [11, 37, 38]. The group from MDACC reported a 10 year PFS of approximately 60% in *IGHV* mutated patients compared to only around 10% for the *IGHV* unmutated

group [11]. Similar analyses were reported by the GCLLSG group as well as in a study by Rossi and colleagues [37, 38]. Thus, it could be argued that the firstline CIT should be reserved only for CLL patients who are *IGHV* mutated [and in the absence of del(17p)].

Minimal Residual Disease (MRD)

Minimal residual disease is an important predictor of long-term outcomes after CIT [11, 39–41]. MRD is typically assessed by flow-cytometry (in either blood or bone marrow) with a sensitivity of 10^{-4}, though more sensitive methods for MRD assessment are being evaluated in clinical trials [42]. MRD assessment is now being incorporated as an endpoint in most clinical trials.

First-Line Therapy by Patient and Disease Status

1. "Young fit" patients [without del(17p)]: The German CLL Study Group (GCLLSG) has defined these individuals as having low comorbidity score (CIRS <6) and adequate renal function (creatinine clearance greater than or equal to 70 ml/min). In our practice, we typically consider patients less than 65 years of age with good performance status and good organ function in this age group. For these patients, treatment with CIT is considered standard. Given favorable long-term outcomes for *IGHV*-mutated patients, we consider this group appropriate for CIT. FCR is our CIT of choice (for patients >65 years and in those with poor renal function – BR is preferred over FCR). For patients with unmutated *IGHV*, we recommend treatment with novel agents in clinical trials as long-term outcomes with CIT remain suboptimal.
2. Older adult patients or those with significant comorbidities [without del(17p)]: These patients are deemed ineligible to tolerate FCR/BR regimens. Until recently, the combination of chlorambucil and obinutuzumab was the preferred treatment choice, based on the results of the CLL11 trial. With the results of the RESONATE-2 trial (see "BTK inhibitors" section) showing superiority of ibrutinib over chlorambucil, we believe ibrutinib should be considered standard treatment for this group of patients.
3. Patients with del(17p): These patients, irrespective of age, should be considered for ibrutinib therapy (see "BTK inhibitors" section) given the poor outcomes with CIT for this group of patients.

Novel Targeted Therapies

Several novel targeted therapies have been developed for treatment of patients with CLL including B cell receptor (BCR) inhibitors (Bruton's tyrosine kinase (BTK) inhibitors, PI3-kinase (PI3K) inhibitors), BCL-2 inhibitors, the immunomodulatory drug lenalidomide, and more recently immunotherapy with chimeric antigen receptor (CAR) T cell therapy.

BTK Inhibitors BTK is a non-receptor tyrosine kinase that has an important role in BCR signaling and in CLL cell survival. Ibrutinib is an oral, selective, and irreversible inhibitor of BTK, which binds to the cysteine-481 site of BTK. Ibrutinib was FDA approved for treatment of CLL in 2014, initially in the relapsed setting, and in 2016 the approval was expanded to the first-line setting. The standard dose of ibrutinib for patients with CLL is 420 mg orally once daily. In the initial phase I–II trial, 101 patients with relapsed/refractory (R/R) CLL were treated with ibrutinib single agent [43]. The median age was 64 years, with 34% patients with del(17p) and additional 35% with del(11q). These patients were a heavily pretreated group with a median number of prior therapies of 4 (range, 1–12). In a recent update, after a median follow-up of 49 months, an ORR of 89% (76% PR, 10% CR) was noted [44]. The median PFS was 52 months (5-year PFS 43%). The median PFS for patients with del(17p) and del(11q) was 26 months and 55 months, respectively. The median PFS was not reached for the other FISH subgroups. In this study, 31 patients with treatment-naive CLL were also treated with ibrutinib. The median age was 71 years (range, 65–84). After a median follow-up of 62 months, the ORR was 87% with a CR rate of 29%. The 5-year PFS was 92%. It is important to note that most patients will develop reactive lymphocytosis after initiation of ibrutinib, which peaks within 1–2 months and gradually resolves over the course of the next 6–9 months. This situation is not disease progression, and patients should be continued on ibrutinib. Neither the occurrence of reactive lymphocytosis nor the degree or duration of lymphocytosis has any effect on the PFS of patients treated with ibrutinib. The most common toxicities with ibrutinib are diarrhea, easy bruising, skin rash, arthralgia, and hypertension. Atrial fibrillation occurs in approximately 8–10% of patients treated with ibrutinib; this frequency is approximately four times that of the control arm in randomized controlled trials of ibrutinib [45].

In a phase III trial (RESONATE trial), patients with R/R CLL were randomized to ibrutinib (n = 195) vs. ofatumumab (n = 196). Ibrutinib led to a superior ORR, as well as a longer PFS and OS compared to that seen with ofatumumab [46]. In the first-line setting, ibrutinib was compared to chlorambucil in patients 65 years or older [47]. The ibrutinib arm produced a superior PFS and OS. The 2-year PFS was 89% for ibrutinib vs. 34% for chlorambucil ($p < 0.0001$). This trial showed an OS advantage with ibrutinib in an intent to treat analyses even after crossover was allowed for those progressing on chlorambucil.

Mechanisms of resistance to ibrutinib are an area of active investigation. The majority of patients who fail ibrutinib have a mutation of *BTK* at cysteine-481 (C481S) [48]. Less commonly, a gain-of-function mutation in *PLCγ2*, a signaling molecule downstream of BTK, is noted.

Acalabrutinib is a more specific BTK inhibitor than ibrutinib and has shown clinical activity in patients with CLL [49]

and recently was approved as monotherapy in R/R mantle cell lymphoma. An ongoing randomized phase III trial is comparing acalabrutinib vs. ibrutinib in high-risk R/R CLL. Several other BTK inhibitors are in clinical development [50].

PI3-K Inhibitors Of the several isoforms of PI3-Kinase, PI3K-delta is important for proliferation and survival of B cells [51]. Idelalisib is a selective, reversible inhibitor of PI3K-delta. In a phase I study in patients with R/R CLL, 54 patients were enrolled [52]. The median number of prior therapies was 5. The ORR was 72% with a median PFS of 15.8 months. A phase III trial comparing idelalisib + rituximab versus placebo + rituximab in R/R CLL showed improved PFS and OS in the idelalisib arm [53]. Based on this trial, the FDA approved the combination of idelalisib and rituximab for patients with R/R CLL where rituximab monotherapy would be considered appropriate. Idelalisib has also been studied in the first-line setting in combination with rituximab; however, diarrhea/colitis developed in a majority of the patients [54]. In another study, patients received idelalisib with ofatumumab for first-line therapy of CLL [55]. Immune-mediated hepatotoxicity, due to downregulation of T regulatory cells, developed in the majority of the patients. Patients who receive treatment with idelalisib should receive PCP prophylaxis and need to be monitored for CMV reactivation. Besides idelalisib, several other PI3-K inhibitors are in clinical development in CLL, including duvelisib (IPI-145) (PI3-K gamma and delta inhibitor) and umbralisib (TGR-1202) (PI3-K delta inhibitor).

BCL-2 Inhibitor

There is high expression of Bcl-2 family member anti-apoptotic proteins in CLL cells, making BCL2 a potential therapeutic target. Venetoclax is a specific inhibitor of BCL-2 [56] and is currently approved for patients with del(17p) CLL who have received prior therapy. Due to a risk of tumor lysis, venetoclax is administered in a weekly dose escalation schedule, starting at 20 mg once daily up to a target dose of 400 mg daily. In a phase I study, a total of 116 patients with R/R CLL (median number of prior therapies = 3) were treated (dose-escalation, $n = 56$; dose expansion, $n = 60$) [57]. In the dose-escalation phase, patients received venetoclax in doses ranging from 150 to 1200 mg per day. In the expansion phase, patients received weekly stepwise ramp-up to the target dose of 400gm daily. An ORR of 79% with a CR rate of 20% was noted. Clinical tumor lysis occurred in three patients in the dose escalation phase with no clinical tumor lysis in the dose expansion phase. Grade 3–4 neutropenia occurred in 41% of the patients. Venetoclax in combination with rituximab was investigated in R/R patients with CLL ($n = 49$) [58]. The median number of prior therapies was 2. Overall, 86% had a response; the CR rate was 51%. MRD negativity in the marrow was achieved in 57% of the patients. Eleven patients who had MRD negative remissions discontinued venetoclax and remained MRD negative with a median follow-up of 9.7 months. In a phase III trial in R/R CLL (median prior therapy = 1), venetoclax + rituximab (VR) was compared to BR [59]. The primary endpoint was investigator-assessed PFS. A total of 389 patients were enrolled. The median age was 64.5 years and the median number of prior therapies was 1. The PFS was superior for the VR arm (HR 0.17, $p < 0.0001$; median not reached vs. 17.0 months). The PFS estimates at 2 years were 84.9% vs 36.3%, respectively. Besides PFS, there was significant improvement in ORR, CR rate, and OS for the VR arm. G3–4 neutropenia was noted in 57% of the patients in the VR arm.

Lenalidomide

Lenalidomide is an immunomodulatory drug with multiple immune effects on the tumor microenvironment [60]. In R/R CLL, lenalidomide monotherapy produced an ORR of 30–45% with approximately 10% of patients achieving CR, including MRD-negative remissions [61, 62]. Lenalidomide has also been investigated as initial therapy for CLL [63, 64]. An ORR of 65% with 10% CR rate was noted. Tumor flare and tumor lysis syndrome have been noted with lenalidomide use. Studies have also investigated combinations of lenalidomide with CD20 mAb.

Chimeric Antigen Receptor (CAR) T Cell Therapy

A CAR is a recombinant protein composed of an antigen-binding domain derived from single chain immunoglobulin variable genes, an intracellular signaling domain derived from CD3ζ and costimulatory domains derived from CD28 and/or CD137. For the autologous CART cells, patients undergo leukapheresis to collect T cells. The T cells are genetically modified to add the CAR receptor targeting a specific antigen, most commonly CD19. These engineered T cells are infused into the patient, generally after lymphodepletion chemotherapy, usually fludarabine and cyclophosphamide. These T cells bind to the target antigen on the tumor cells, cause cell lysis, cytokine production, and T cell activation. Early clinical data is promising, and long-term outcomes are awaited [65, 66]. Turtle and colleagues reported results with JCAR014 in patients with R/R CLL [67]. A total of 24 patients were treated. The median number of prior therapies was 5. All patients had received prior ibrutinib. FDG-avid disease was noted in 93% of the patients who had a PET scan. After 4 weeks of CART infusion, an ORR of 74% with a CR rate of 21% was seen. The majority (88%) of the patients were MRD negative by flow cytometry

in the marrow at 4 weeks. Of the patients with PET-avid disease, 64% were in CR at 4 weeks.

Hematopoietic Cell Transplantion

Allogeneic hematopoietic cell transplantation (allo-HCT) remains a potentially curative modality for patients with CLL. However, with the introduction of novel targeted therapies, the role of allo-SCT has declined. Allo-HCT leads to 5-year event-free survival (EFS) and OS rates of 35–45% and 50–60%, respectively [68]. In the targeted therapy era, allo-HCT should be considered for patients with del(17p) who have failed at least one targeted therapy such as ibrutinib or venetoclax.

Autoimmune Complications of CLL

Patients with CLL can develop autoimmune complications. Autoimmune hemolytic anemia (AIHA), immune thrombocytopenia (ITP), and pure red cell aplasia (PRCA) occur in less than 10% of patients with CLL [69]. The autoantibodies in CLL are typically polyclonal, indicating that they are not produced by the leukemic clone. Steroids are generally the initial treatment of AIHA and ITP. Though corticosteroids lead to a remission in the majority of cases, relapses are common. Rituximab is commonly added to steroids for the initial treatment. IVIG can also be used as an adjunctive treatment [70]. Cyclosporine is another option for treatment of immune-mediated cytopenias [71]. Splenectomy is rarely used these days but can be considered in refractory cases.

Hypogammaglobulinemia

Hypogammaglobulinemia is a frequent complication of CLL and predisposes patients to recurrent infections, especially sinopulmonary infections. In a randomized study, IVIG was shown to decrease the incidence of bacterial infections [72]. Replacement IVIG treatment is indicated for patients with hypogammaglobulinemia and severe repeated infections [70].

Richter Transformation

Richter transformation (RT) represents CLL transformation to an aggressive lymphoma, most commonly diffuse large B-cell lymphoma (DLBCL), and rarely to Hodgkin lymphoma transformation [73]. RT occurs in approximately 5% of patients with CLL. Clinically, patients may present with worsening B symptoms, rising LDH, and rapid lymph node enlargement (many times localized). It is important to obtain a PET scan whenever RT is suspected and to biopsy the area with the highest SUV [74]. Whenever available, it is important to assess the clonality of the Richter tissue (by *IGHV* sequencing) and compare it to that of the underlying CLL. In approximately 80% of patients with DLBCL transformation, the DLBCL is clonally related to the original CLL [75]. These patients have the worst outcomes with a median survival of <1 year. In patients with clonally unrelated RT (20% of the cases), the DLBCL clone may represent a new malignancy, and the outcome is similar to that of de novo DLBCL. Several studies in the CIT era have evaluated risk factors for development of RT [76]. These risk factors include lymph node >3 cm, number of prior therapies, advanced Rai stage (III-IV), FISH abnormalities such as del(17p) and del(11q), unmutated *IGHV* gene, short telomere length, stereotyped B cell receptors, expression of CD38, CD49d, ZAP-70, and *NOTCH1* mutation [77]. With the use of ibrutinib and venetoclax, complex karyotype has been associated with the development of RT. Studies looking at the biology of RT in the context of CIT have shown that activation of *C-MYC*, inactivation of *CDKN2A*, and *NOTCH1* mutations are important genetic events [78, 79]. The conventional treatment of RT is with intensive chemoimmunotherapy such as OFAR (oxaliplatin, fludarabine, cytarabine, and rituximab), Hyper-CVAD, and R-CHOP [80]. However, the response rates are around 40% and median survival is only 8–9 months. Allo-SCT remains a potentially curative option for patients with RT; however, less than 10% of patients with RT are able to proceed to transplant. Recent clinical trials are evaluating the role of checkpoint inhibitors and CART cellular therapy in patients with RT [67, 81, 82].

Summary

The treatment of CLL has undergone a remarkable evolution in the last few years. With the introduction of targeted therapies, the role of chemotherapy has declined. Several ongoing randomized trials will help further refine the treatment strategies for patients with CLL.

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Plasma Cell Disorders

34

Taimur Sher and Morie A. Gertz

Introduction

Plasma cell disorders (PCD) also known as dysproteinemias or plasma cell dyscrasias are a group of related diseases arising from a common progenitor belonging to the B-lymphocyte lineage. These disorders are characterized by the expansion of plasma cells in the bone marrow (BM) and/or other lymphoid tissue. This group of diseases includes monoclonal gammopathy of undetermined significance (MGUS), multiple myeloma (MM), Waldenstrom macroglobulinemia (WM), and immunoglobulin light chain (AL) amyloidosis among others (Table 34.1). A unique feature of these disorders is the presence of a "tumor marker" monoclonal immunoglobulin (Ig) or Ig fragment in the serum and/or urine of the patients detected by serum or urine protein electrophoresis (SPEP and UPEP), respectively (Fig. 34.1a, b). The SPEP and UPEP identify the presence of a "monoclonal" Ig as a "spike" known as M-spike (M for monoclonal) that can vary in size from barely measureable to several grams/dL. Immunofixation electrophoresis identifies the type of M protein based on the heavy and light chain isotypes (there are five isotypes for heavy chain G, A, M, E, and D and two for light chains, kappa (κ) and lambda (λ)).

Normal humoral immune response involves the maturation of B-lymphocytes through various stages into Ig- or antibody-secreting plasma cells. This evolution involves rearrangement of Ig heavy (VDJ, on chromosome 14) and light chain (VJ, on chromosome 2 for kappa and chromosome 22 for lambda) gene segments, somatic hypermutations, and Ig class switching. Gene sequence analysis of Ig variable genes of the heavy chains (IgVH) indicates that plasma cells in MM (the most common type of clinically significant plasma cell disease) are derived from post-follicular B-cells that are at the stage of Ig class switching and either are or about to be a memory B-cell. Chromosomal translocations involving immunoglobulin gene segments and other parts of the genome provide an opportunity for the promotor regions of DNA to be brought in close proximity to oncogenic genes which have the potential to initiate or sustain the transformation process in various plasma cell dyscrasias.

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Monoclonal Gammopathy of Undetermined Significance (MGUS)

MGUS is a premalignant plasma cell disorder defined by the presence of a monoclonal protein <3.0 g/dL, <10% clonal plasma cells in the bone marrow, and the absence of plasma cell-related end-organ damage (anemia, proteinuria, lytic bone disease, hypercalcemia, renal disease) [1]. The overall prevalence of MGUS in the USA is 2.4% in individuals over the age of 50 years. The highest prevalence is found in African Americans (3.7%), followed by whites (2.3%) and Mexican Americans (1.8%). MGUS is more common in men, and its prevalence increases with age ranging from 1.2% to 2.8% in 50–59-year-olds to 4.6–8.7% in individuals 80 years or older [2]. The etiology of MGUS is largely unknown, but male gender, African American race, previous autoimmune diseases, and exposure to certain pesticides all have been associated with increased risk of developing MGUS.

There are three major types of MGUS: non-IgM MGUS, IgM MGUS, and light chain MGUS. Non-IgM MGUS is the most common type with IgG or IgA as the M protein. In the case of progression, the majority of such cases transform to smoldering myeloma and multiple myeloma. IgM MGUS is less common and is defined as a monoclonal IgM protein <3.0 g/l, <10% bone marrow lymphoplasmacytic cells, and the absence of end-organ damage (anemia, hyperviscosity, lymphadenopathy, hepatosplenomegaly, constitutional symptoms) attributed to a lymphoproliferative disorder. It can progress to smoldering WM, clinically overt WM, and, rarely, IgM myeloma, chronic lymphocytic leukemia, or

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Table 34.1 Comparison of most common PCD

Characteristic	MGUS	MM	AL	WM
Incidence	Very common	Most common PC malignancy	Rare. 8 in a million	Rare. 3 in a million
Clinical presentation	Asymptomatic	C: Hypercalcemia	Heart failure	B-symptoms, lymphadenopathy
		R: Renal failure	Liver enlarge	Hyperviscosity
		A: Anemia	Neuropathy	Neuropathy
		B: Bone disease	Nephrosis	Cryoglobulinemia
			Bleeding	Anemia
Bone marrow findings	Less than 10% plasma cells	>10% plasma cells. Frequently more than 40%. Can be very proliferative disease as in plasma cell leukemia	Plasma cells are non-proliferative typically 5–7% plasma cells in marrow	Usually diffuse disease. Has to be more than 10% in order to be called WM
Imaging findings	Normal	Lytic bone disease	Echocardiogram and MRI show cardiomyopathy	Lymphadenopathy. If very active lesion on PET scan, consider transformation to high-grade lymphoma
		MRI and PET scan show active lesions. PET scan can show extramedullary disease	The liver and spleen can be enlarged	
Unique features	Recognize association with kidney disease and neuropathy	Bone disease and renal disease are common	Diagnosis is frequently delayed	Hyperviscosity syndrome can be common and has high morbidity and mortality if not promptly recognized
			It is imperative to determine the type of amyloid with proteomic testing as patients with non-light chain amyloidosis (transthyretin amyloidosis is the most frequent one) can have co-existing MGUS and be treated un-necessarily with chemotherapy	Rituximab-induced Ig M flare is common and may need plasma exchange to control blood viscosity
			Multiple organ involvement is very common, making it the PCD with severe symptom burden and high morbidity and mortality	
			Close attention should be paid to unique clinical features of macroglossia, periorbital purpura, and shoulder fat pad sign	
			A patient with heart failure with preserved ejection fraction has amyloid cardiomyopathy until proven otherwise	

Table 34.1 (continued)

Treatment	In itself does not need treatment. It is important to recognize associated conditions such as MGUS of renal significance and MGUS-related neuropathy as these can lead to significant morbidity if underlying plasma cell clone is not treated	Smoldering multiple myeloma does not require treatment outside the setting of clinical trials	Prompt treatment is very important	Rituximab in combination with an alkylating agent (BR +/− steroids RCD) or rituximab plus bortezomib is the appropriate initial treatment
		Initial treatment consists of PI and IMiD and a steroid triplet. ASCT is an important strategy to improve the depth of response in patients who are candidates for this modality	Patients who are candidates for ASCT and have less than 10% plasma cells in the marrow can be treated with primary ASCT	Btk inhibitor ibrutinib is an effective treatment and can cause rapid reduction in IgM
		Maintenance treatment results in improved PFS and more recently has shown to improve OS	Majority of patients (> 75%) are not candidates for ASCT, and treatment with either melphalan or dexamethasone or bortezomib-based regimen should be started. This disease responds very well to systemic therapy. The ultimate goal of treatment is organ improvement	Complete responses in WM are less common as compared to other plasma cell dyscrasias
		Daratumumab is likely to move into frontline setting and improve the outcomes across all lines of therapy	Supportive care is the cornerstone of management	
		Cellular immunotherapy with CAR-T cells is very promising, and larger studies are eagerly awaited		
		Supportive care for bone disease, anemia, cytopenias, renal disease, and neuropathy is very important		
Prognosis	Depends on the natural history and associated conditions	Highly dependent on patient age and cytogenetic risk of myeloma	Extent of cardiac involvement determines the prognosis. Median survival for patients with early-stage disease is in excess of 5 years and that of advanced stage disease is less than 6 months	Prognosis is dependent on age, degree of marrow compromise, beta-2 microglobulin levels, and IgM levels. Based on these 5-year survival in low- and high-risk patients is 87% and 36%, respectively
		Significant improvement in overall survival with average survival of average risk myeloma being 10 years or longer		
		Patients with 17p deletion have a median survival of 3 years or less		

Abbreviations: PCD plasma cell dyscrasia, *MM* multiple myeloma, *WM* Waldenstrom macroglobulinemia, *MGUS* monoclonal gammopathy of undetermined significance, *AL* immunoglobulin light chain amyloidosis, *CAR-T* chimeric antigen receptor modified T-cells

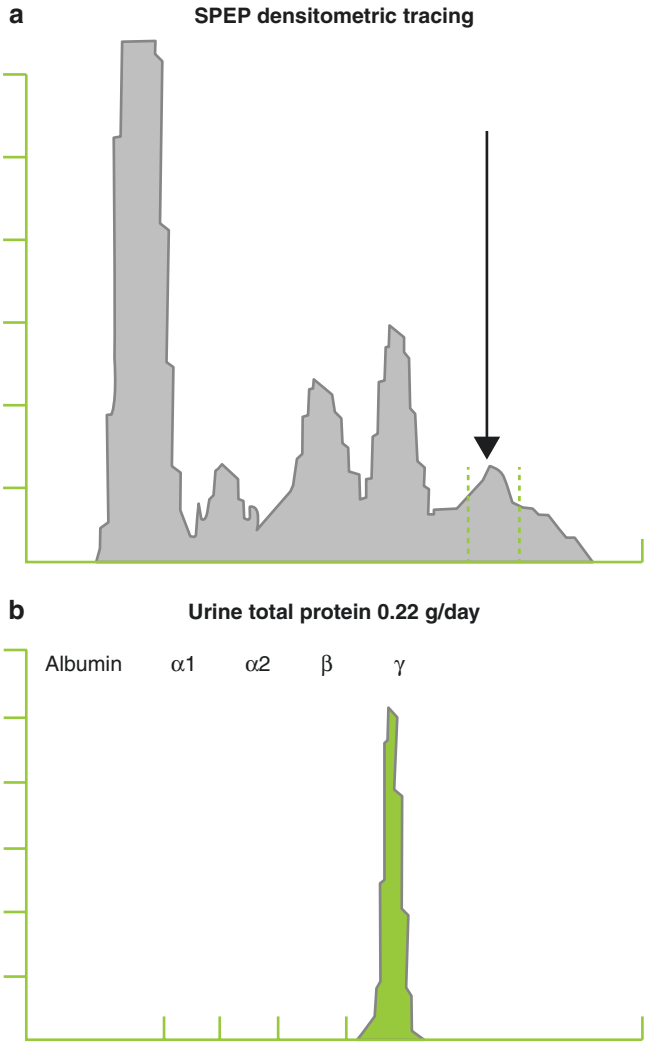

Fig. 34.1 (**a**) Serum protein electrophoresis in a patient with light chain myeloma shows a small abnormality in the gamma region. (**b**) Urine protein electrophoresis in a patient with MM

other B-cell non-Hodgkin lymphomas. Light chain MGUS (LC-MGUS) is a relatively recently described entity and represents approximately 20% of all MGUS cases. LC-MGUS is defined as abnormal serum free light chain ratio (FLC) (<0.26 or >1.65) in combination with elevated concentration of the *involved* κ or λ light chains. LC-MGUS can progress to light chain myeloma and light chain amyloidosis (AL) [3].

In the majority of patients, MGUS is diagnosed incidentally during evaluation for various symptoms or disorders. The risk of progression from MGUS to myeloma and other lymphoproliferative disorders is approximately 0.5–1% per year and persists throughout follow-up; hence, it is important for these patients to be continuously monitored. The risk is not uniform, and several risk-scoring systems have been devised to identify patients at higher risk of transformation. Using an abnormal FLC ratio (<0.26 or >1.65), M protein level >1.5 g/dL, and reduction of one or two uninvolved immunoglobulin isotype levels (immunoparesis), the National Cancer Institute and Nordic Myeloma Study Group (NCI/NMSG) have identified a group of MGUS patients (those with all three risk factors) with a 10-year risk of disease progression of 40% [4].

Long-term follow-up of large clinical cohorts has revealed the association of MGUS with a number of comorbidities outside of its "malignant potential." These include increased risk of infections, venous and arterial thrombosis, increased bone turnover, and an elevated risk of fractures. There is an increased risk of renal complications in patients with MGUS, especially LC-MGUS, and the term monoclonal gammopathy of renal significance has been coined to describe patients who have M protein-related renal diseases such as monoclonal immunoglobulin deposition disease, light chain proximal tubulopathy, and proliferative glomerulonephritis with monoclonal immunoglobulin deposits. These renal diseases are characterized by deposition of monoclonal deposits in the kidney diagnosed on kidney biopsy. In addition, the M protein can cause various systemic manifestations including neuropathy and skin disorders. It is important to thoroughly pursue these associations as they may require treatment even in the absence of "malignant" plasma cell disorders such as MM and WM making such cases of MGUS as "not so insignificant" [5].

Multiple Myeloma

Epidemiology

Multiple myeloma (MM) is the most common cancer of plasma cells and after non-Hodgkin lymphoma the second most common hematological malignancy accounting for 13% of all blood cancers, approximately 2% of all cancer deaths, and 20% of deaths caused by hematological malignancies. In 2017 an estimated 30,280 new cases of MM were diagnosed in the USA with the annual age-adjusted incidence being 6.6 cases per 100,000 people. MM is typically a disease of the elderly, median age at diagnosis is approximately 70 years, 37% of patients at diagnosis are less than 65 years, 27% are aged 66–75 years, and 37% of patients are over 75 years [6].

The etiology of MM remains unknown. History of MGUS, African American race, and advanced age are the greatest risk factors. Exposure to pesticides and herbicides including Agent Orange (dioxin) has been associated with an increased risk of developing MM [7].

The diagnosis of MM requires the presence of at least 10% of monoclonal plasma cells in the bone marrow biopsy. MM can be asymptomatic (smoldering), when no myeloma-related organ or tissue dysfunction is present, or symptomatic, when the so-called CRAB criteria are present: hypercalcemia, renal failure, anemia, and bone disease. In 2014, the International Myeloma Working Group (IMWG)

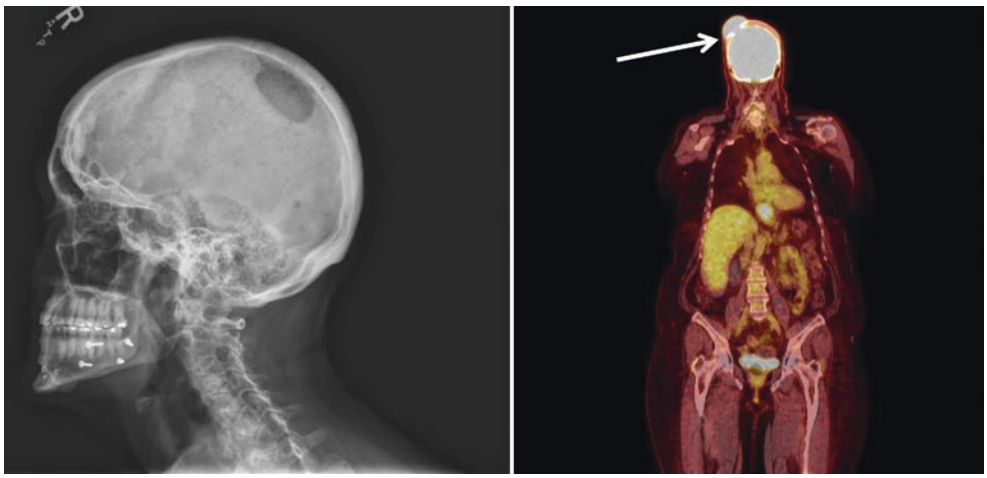

Fig. 34.2 Skull X-ray showing a large lytic defect (on left) that can be better seen in the PET scan (on right, see arrow). The patient presented with a rapidly enlarging tumor on the parietal region and biopsy demonstrated sheets of plasma cells. This is consistent with a plasmacytoma arising out of the skull

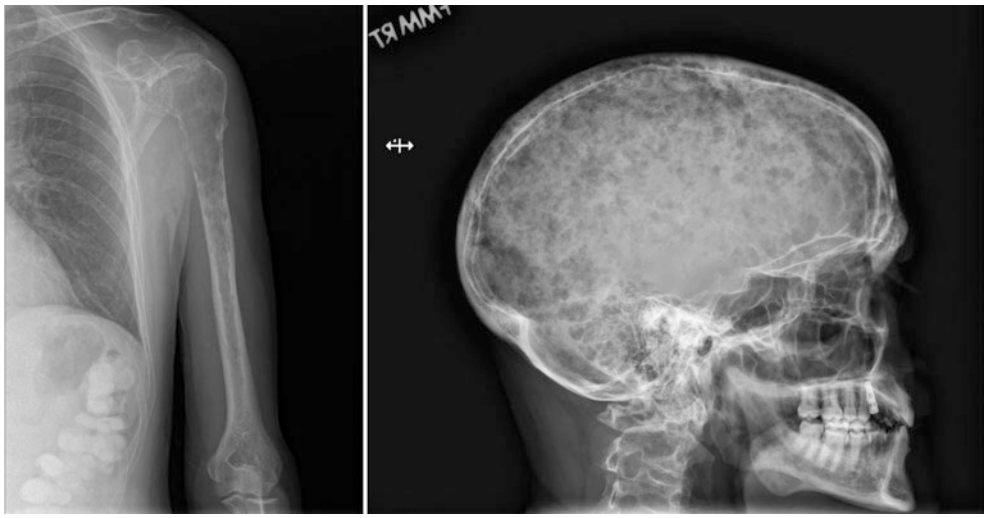

Fig. 34.3 Skeletal survey of a MM patient with advanced bony disease. Complete replacement of humeral head with tumor can be seen on the left. Multiple punched out lesions on the skull give rise to the classic "salt and pepper" appearance (on the right)

proposed an updated criteria for diagnosis of symptomatic/treatment requiring MM and 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) to the original CRAB criteria [8]. The rationale behind the updated definition is twofold; firstly, there are multiple effective agents available for treatment of MM, and secondly, early therapy in asymptomatic high-risk patients could potentially reduce the risk of end-organ damage and prolong survival by preventing the transformed clone from acquiring genetic alterations typical of aggressive and advanced disease.

Pathophysiology

Fluorescent in situ hybridization (FISH) studies of MM have recognized two distinct subgroups: those with chromosome 14 translocations (*IgH* locus) and others with hyperdiploidy. Interestingly, studies have shown that these changes are already present in MGUS, indicating these to be early "hits" in myeloma pathogenesis [9]. So far, no single disease-specific gene has been identified. Modern sequencing techniques such as whole genome, exome, and targeted sequencing have provided interesting insights into the genetic landscape of MM and revealed complex alterations that include frequent somatic mutations in *KRAS*, *NRAS*, *FAM46C*, *BRAF*, *TP53*, *TRAF3*, *DIS3*, *CYLD*, and others. Progression from MGUS to myeloma may be caused by acquisition of additional genetic events or the expansion of pre-existing 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 [10].

MM is the most frequent cancer to involve the bone with almost 70% of patients presenting with bone lesions at diagnosis [11]. In some series up to 20% of patients present with a pathological fracture at diagnosis, and a majority (close to 60% of patients) develop a pathological fracture over the course of their disease (Figs. 34.2 and 34.3) [12]. Spinal cord compression from a myeloma deposit (plasmacytoma) or pathological fracture can be the initial presentation and

be a source of significant long-term morbidity. Healing is generally poor, and MM patients continue to experience the sequelae of bone disease in the long term, even with good control of the plasma cell burden. Myeloma bone disease results from the interaction of MM cells with osteoclasts and bone marrow microenvironment. Such interactions result in feedback loops that sustain MM cells and tip the balance of bone turnover toward reabsorption. These interactions are mediated by cytokines and humoral factors such as RANK ligand, a highly potent osteoclast-stimulating factor; macrophage inflammatory protein-1 alpha; and interleukin (IL)-3 and IL-6 production by marrow stromal cells, MM cells, osteoclasts, and other cells in the bone microenvironment [13].

Clinical Manifestations and Workup

Fatigue and pain from anemia and bone disease are the most common symptoms, seen in approximately 80% of patients. Confusion, constipation, and dehydration can be seen in patients with hypercalcemia and renal failure. Rare presentations include bleeding and thrombosis as a result of anti- and procoagulant properties of M proteins. Patients with highly aggressive and proliferative disease can present with disseminated intravascular coagulopathy [14]. Symmetric peripheral sensory neuropathy from axonal degeneration can also be the presenting feature. Symptoms of hyperviscosity can be present in patients with extreme elevations of M protein. Patients with extramedullary disease can present with tumors involving soft tissues in the paraspinal and head and neck region. Skin involvement by plasmacytomas is uncommon as an initial presentation but can be seen in patients with advanced disease. Immunoglobulin-related cast nephropathy, hypercalcemia, dehydration, and non-steroidal anti-inflammatory drugs frequently used by the patients to manage pain all contribute to myeloma renal disease that is seen in 20–25% of patients [15].

Laboratory abnormalities are common and include the presence of a serum and/or urine M protein in approximately 80% of cases. Light chain is the only secretory protein in 20% of patients and an SPEP will not reveal an M-spike in such patients. Nonsecretory myeloma, where SPEP, UPEP, and sFLC fail to show a secretory component, is rare and is seen in 1–2% of patients. Workup of a multiple myeloma patient includes evaluation of major organ system function, imaging studies, and bone marrow examination (Table 34.2).

Treatment of Multiple Myeloma

Untreated MM is fatal and is associated with severe morbidity related to bone disease, renal disease, infections, and bone marrow failure. The treatment of multiple myeloma has seen a revolution over the last 20 years with remarkable improvement in outcomes [16]. Up until the early to mid-1990s, melphalan and steroids were the mainstay of treatment. Currently there are more than ten medications approved by the US Food and Drug Administration (FDA) for the treatment of MM, and many more are in the pipeline. The following discussion pertains to general principles of management. Medications commonly used to treat MM and other plasma cell dyscrasias are listed in Table 34.3.

MM is an incurable malignancy and the majority of patients are elderly. Age, medical comorbidities, and myeloma risk profile are the three most important of the several patient- and disease-related factors taken into consideration to decide on the best treatment. Treatment involves antineoplastic therapy directed against the malignant plasma cells and supportive care to address the disease manifestations. Antineoplastic treatment involves three stages (initial or induction therapy, consolidation, and maintenance treatment). Proteasome inhibitors (bortezomib, carfilzomib, and ixazomib) and immunomodulatory drugs (thalidomide, lenalidomide, and pomalidomide) in combination with steroids form the backbone of initial myeloma therapy. Responses with the three-drug combination can be achieved in more than 90% of patients. Initial treatment typically is given for 4–6 months and then the patient is assessed for optimal consolidation therapy. Patients who are not eligible for high-dose chemotherapy and autologous peripheral blood cell rescue also known as autologous hematopoietic cell transplant (AHCT) continue the initial treatment in the consolidation phase for at least 8–12 months of treatment with the aim being to achieve deep and durable remission (very complete response or very good partial response). Patients who are candidates for AHCT have their progenitor cells mobilized and harvested and undergo myeloablative chemotherapy with high-dose melphalan after which the collected cells are reinfused. An important part of management has been to identify the patients fit enough to undergo AHCT; historically, it was important to determine this eligibility prior to initiating induction therapy as many treatments had the potential to interfere with stem cell harvesting, but with newer treatments, this is no longer a concern, and the main reason to determine this eligibility is to improve the therapeutic index of the consolidation regimen by selecting fit and healthy patients for AHCT. In Europe patients older than 65 years, generally are not offered AHCT. In the USA, this decision is based more on the "physiologic age" as determined by the overall functional status, health, and organ system function evaluation of the patient. Several tools exist to help determine the eligibility of patients such as Charlson comorbidity index and the hematopoietic cell transplantation comorbidity index (HCT-CI). AHCT does not result in cure but increases the depth of response, and recent evidence

Table 34.2 Tests and procedures for evaluation of patients with PCD

Test/procedure	Comments
Complete blood count (CBC)	Simple and cheap test to assess the extent of bone marrow involvement
	Hb has prognostic value for WM
	Hemolytic anemia can be seen in WM
	Important to monitor treatment toxicity
Simple chemistry (renal and hepatic function tests and electrolytes)	Vital in assessing the disease and its impact on various organs
	Hypercalcemia and renal insufficiency are very important component of treatment decision for MM
	Hypoalbuminemia is often the first presenting sign of amyloidosis-related nephrotic syndrome
	Liver and kidney functions inform about the extent of disease in WM
	Bilirubin and creatinine values help guide treatment monitoring and dose modifications for antineoplastic medications
Beta-2 microglobulin	Very important prognostic marker in multiple myeloma and increasingly in WM
	In combination with serum albumin provides the basis for international staging system (ISS)
	Can be elevated in patients with renal failure
SPEP and UPEP with immunofixation electrophoresis (IFE)	Basic screening tests for plasma cell disorders to detect the tumor marker
	IFE is more sensitive than SPEP and is the only test used to give a name to PCD, e.g., IgA kappa MM, lambda light chain MM
Serum free light chain assay	Very important test to diagnose light chain only MM, AL amyloidosis, and light chain deposition disease
	Incorporated into the new definition of treatment requiring MM
	Is used to prognosticate and monitor response to treatment in AL
	Values can be affected by creatinine clearance
Troponin T and NT-ProBNP	Biomarkers of cardiac damage in patients with AL amyloidosis
	The most important predictors of survival and suitability for stem cell transplantation in AL
Bone marrow examination	Vital to make diagnosis of MM and WM
	Extent of bone marrow plasmacytosis is prognostic in patients with AL and helps determine the appropriate initial treatment of patients who are transplant eligible. Those with less than 10% plasma cells typically are treated with SCT as initial treatment as compared to others who are given "cytoreductive" standard chemotherapy prior to SCT
	Cytogenetics inform about the risk stratification and has emerged as the single most important prognostic factor in MM
	Critical to assess the depth of response to treatment
	It is not needed in all patients with MGUS. If all of the following are met, it is very reasonable to monitor patients closely without bone marrow at initial diagnosis
	Asymptomatic patient without organ damage (blood, urine, and imaging studies are normal)
	M-spike is less than 1 g/dL and of IgG type
	Serum free light chains are normal
Imaging studies	
Skeletal survey	Cheap, readily available, and fast test to determine myeloma bone disease
	Does not inform about "early bone disease" and extramedullary disease
	Great test to assess the extent of marrow involvement and access the number of lesions
Magnetic resonance imaging (MRI)	Ideal to assess spinal cord compromise
	Can help identify AL cardiomyopathy
	Increasingly being used to make early diagnosis of MM
	Can detect extramedullary disease
Positron emission tomography (PET)	Can help follow response to treatment in MM as responding areas of disease become metabolically inactive

suggests that it results in improved progression-free survival [17]. Several studies have demonstrated that addition of the immunomodulatory drug lenalidomide after consolidation therapy improves progression-free survival [18]. A recent meta-analysis has demonstrated that such a strategy also improves the overall survival of MM patients and hence has been incorporated into current practice.

The duration of response achieved with frontline therapy depends largely on the cytogenetic risk of multiple myeloma (Tables 34.4 and 34.5). Patients with high-risk disease (those with 17p deletion and t(14;16)) continue to have a median overall survival of less than 3 years despite very aggressive therapy including the use of triple drug combination after ASCT [19]. Almost all patients experience disease relapse, and further therapy depends upon the pattern of relapse, type of initial therapy, duration and depth of remission, the overall functional status, and medical comorbidities of the patient. Newer treatments with monoclonal antibody-based regimens

Table 34.3 Antineoplastic medications for the treatment of PCD

Drug(s)	Comments
Alkylating agents	
Melphalan	Conventional chemotherapeutic
Cyclophosphamide	Have been in use for more than 5 decades
Bendamustine	Almost always in combination with other agents
	High-dose melphalan is used as conditioning regimen for ASCT
	Secondary myelodysplastic syndrome can result from long-term use of melphalan
Steroids	
Dexamethasone	Very important component of all double and triple drug combinations
Prednisone	Very important in the management of spinal cord compression, renal failure, and hypercalcemia
	Steroid-induced diabetes, mood changes, and weight gain are common side effects
Immunomodulatory drugs	
Thalidomide	Thalidomide ushered in the era of novel agents
	Thalidomide is not commonly used in the USA as it is associated with neuropathy and cardiotoxicity
Lenalidomide	Lenalidomide is the most common IMiD used in the USA. Its clearance decreases in patients with renal dysfunction and as a result increased hematological toxicity is seen
	Lenalidomide is an important component of induction regimen and is frequently used as maintenance therapy
Pomalidomide	Pomalidomide is the most potent IMiD available and is active even in lenalidomide refractory patients
	IMiDs are very well tolerated in MM but can be difficult to use in AL where they can exacerbate heart failure
	Their use in WM is also challenging given the high incidence of treatment-related anemia and cytopenias
	Venous thromboembolism is a common toxicity of all IMiDs. Thalidomide has the highest incidence of neurotoxicity. Lenalidomide causes cytopenias. Pomalidomide has side effect profile in between the two.
Proteasome inhibitors	
Bortezomib	These are one of the most effective anti-plasma cell therapies with broad range of activity against most PCDs
Carfilzomib	In combination with IMiDs and steroids, these are the most commonly used and highly effective combination in MM
Ixazomib	Bortezomib has demonstrated remarkable activity in AL
	In combination with rituximab, bortezomib is an important treatment for WM
	Neuropathy and lymphodepletion are important side effects of bortezomib
	Pulmonary and cardiac toxicity are more common with carfilzomib, while the incidence of neuropathy is lower
	Gastrointestinal side effects are common with all but particularly with ixazomib since it is an oral drug
Monoclonal antibodies	
Daratumumab	The first in class anti-CD 38 antibody. Highly effective in MM and AL
	Optimal activity is in combination with IMiDs
	Can decrease the validity of Coombs test
	Anti-SLAMF7 monoclonal antibody
	Modest activity as single agent
Elotuzumab	Is approved in combination with lenalidomide and dexamethasone
	Less active than daratumumab
Histone deacetylase inhibitor	
Panobinostat	Approved for treatment of MM in combination with bortezomib and dexamethasone
	Not commonly used due to other effective salvage therapies and multiple clinical trials available
	Gastrointestinal toxicity and fatigue are very common side effects

Table 34.4 Risk stratification systems in multiple myeloma

rISS (revised international staging system)
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%

(anti-CD38 antibody daratumumab) are already improving the outcomes of patients with relapsed multiple myeloma, and this drug is being tested as frontline treatment and is expected to become a backbone of therapy. Recently, immune-checkpoint inhibitors (PD-1 inhibitors, PDL-1 inhibitors) have revolutionized the treatment of several malignancies including Hodgkin lymphoma. They are actively being investigated in multiple myeloma and their role is currently uncertain. Lastly, genetic modification of autologous T-cells with chimeric antigen receptors (CAR-T)

Table 34.5 mSMART (Mayo Stratification for Multiple Myeloma and Risk-Adapted Therapy)

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

CA cytogenetic analysis, *GEP* gene expression profile, *PCLI* plasma cell labeling index

has ushered a fundamental change in cellular immunotherapy and is already clinically available for the treatment of ALL and non-Hodgkin lymphoma. Early clinical experience utilizing CAR-T cell therapy against BCMA (B-cell membrane antigen) in multiple myeloma is very positive with responses seen in a majority of patients who have experienced multiple disease relapses on several prior lines of therapies.

Supportive care forms the cornerstone of management of MM. Patients with bone disease are treated with pain control and bisphosphonate therapy. Bisphosphonates have shown to improve skeletal-related adverse events in MM. One of the side effects of bisphosphonates in osteonecrosis of the jaw (ONJ) that has high morbidity can be prevented by maintaining good dental hygiene and avoiding major dental procedures during therapy. Management of kidney disease, neuropathy both from myeloma and its treatment, prophylaxis for venous-thromboembolic disease with immunomodulatory drugs, and appropriate antimicrobial prophylaxis are critical aspects of management of MM.

The survival of MM patients has doubled in what is now known as the era of novel agents (since the introduction of thalidomide in the late 1990s) and continues to improve with remarkable advances in the field of molecular immunology and genetics. In the coming years, monoclonal antibodies are expected to move to frontline of myeloma therapy. Early intervention in high-risk smoldering myeloma is an area of renewed interest. If demonstrated to be durable, the CAR-T cell therapy is expected to move to earlier lines of therapy. With such exciting advances in the field, a cure for myeloma is a reasonable goal.

Waldenstrom Macroglobulinemia

WM, a rare type of plasma cell dyscrasia, is a type of non-Hodgkin lymphoma called lymphoplasmacytic lymphoma (LPL) and is characterized by bone marrow infiltration with lymphocytes with plasmacytic differentiation that produce IgM monoclonal protein. The diagnosis of WM requires the presence of monoclonal IgM and more than 10% lymphocytes with plasmacytic differentiation [20]. The lymphocytes are typically positive for surface IgM and CD20 and they are negative for CD5 and CD23. The plasma cell component expresses the typical plasma cell markers CD138 and CD38. WM can arise from IgM MGUS. Absence of disease-related symptoms and organ damage, less than 10% lymphoplasmacytic cells in the marrow, and IgM levels of less than 3 g/dL are criteria to diagnose a patient with IgM MGUS [21].

With an annual incidence of three per million per year WM is a rare disease that primarily affects older patients (median age 70 years). Unlike myeloma it is predominantly a disease of whites and less than 5% of patients are African American. It is more common in males. The exact etiology is unknown, but association with infections such as hepatitis C is well known. Chronic immune stimulation increasing the possibility of aberrant Ig gene segment recombination may play a role. First-degree relatives of patients with WM have an increased risk of WM and other non-Hodgkin lymphoma. The pathogenesis of WM is relatively less well characterized. Somatic mutations and chromosomal aberrations have been described. A recurrent somatic mutation in MYD 88 L265P is noted in most patients with WM. This is not unique to WM however but increasingly being used to differentiate WM from other indolent non-Hodgkin lymphomas that are difficult to characterize by immunophenotype [20].

Clinical manifestations result from proliferation of neoplastic lymphoplasmacytic cells in bone marrow resulting in anemia. Thrombocytopenia and leukopenia can be present but are less common than anemia. Unlike MM, patients with WM commonly experience lymphoma-related symptoms of lymphadenopathy and organomegaly (liver and spleen enlargement). The monoclonal Ig M can result in a broad spectrum of symptomatology ranging from acute hyperviscosity syndrome that needs urgent medical attention due to risk of vascular stasis (stroke, blindness, acute myocardial infarction, or limb ischemia), to bleeding diathesis, cryoglobulinemia, renal dysfunction, and peripheral neuropathy among others.

Treatment of WM involves the use of the anti-CD20 monoclonal antibody rituximab in combination with alkylating agents (cyclophosphamide or bendamustine) or proteasome inhibitors. The response rates with these regimens range between 60% and 90%, but most of these are partial responses. A unique side effect of anti-CD20 therapy in WM is the risk of IgM flare where IgM levels increase after treatment to decline over time. It is critical to recognize this phenomenon as it can precipitate hyperviscosity symptoms and may require the use of plasma exchange to decrease the serum viscosity. The role of ASCT in the management of WM is less clear. WM cells, like many other B-cells, are dependent on signaling through the Ig receptor complex called B-cell receptor for its survival and interaction with the

microenvironment. Recently, ibrutinib an orally bioavailable inhibitor of Bruton's tyrosine kinase (Btk) has been approved by the FDA for treatment of newly diagnosed and relapsed WM. Btk is involved in signal transduction from BCR to nucleus and has shown remarkable activity in other non-Hodgkin lymphomas such as chronic lymphocytic leukemia, mantle cell lymphoma, and marginal zone lymphoma [22].

Immunoglobulin Light Chain Amyloidosis

Amyloidosis is a unique yet remarkably heterogeneous group of diseases characterized by deposition of misfolded (amyloidogenic) protein precursors in beta-pleated sheet configuration in the extracellular space in various tissues. The diagnosis of amyloidosis must be confirmed by histological examination using Congo red stain. Once a histologic diagnosis is established, the next step should be determination of the amyloid type. The gold standard for determining the type of amyloidosis is detection of amyloidogenic protein in microdissected specimens of involved tissue by immunogold electron microscopy or proteomic analysis by mass spectrometry. The accurate determination of amyloid type is especially important to exclude transthyretin amyloidosis (ATTR) as it can clinically mimic immunoglobulin light chain amyloidosis (AL). Up to 20% of patients with non-AL amyloidosis can have co-existing monoclonal gammopathy of undetermined significance, which can lead to the misdiagnosis of AL in these patients [23].

Immunoglobulin light chain amyloidosis also called primary systemic or AL amyloidosis is the most common systemic amyloidosis in the USA and Western Europe. It is a plasma cell dyscrasia where clonal plasma cells in the bone marrow produce amyloidogenic light chains. These light chains damage organs by mechanism(s) not completely understood but likely involve direct cytotoxicity. Most commonly it occurs as a de novo disease, but in a minority of patients, AL can evolve from pre-existing multiple myeloma (the most common plasma cell malignancy) and other immunosecretory malignancies such as Waldenstrom macroglobulinemia. Cardiac involvement is often very advanced and is seen in up to 70% of AL patients. AL cardiomyopathy presents with restrictive physiology, and patients frequently present with congestive heart failure with a non-dilated, thickened left ventricle on echocardiogram. Peripheral edema from right heart dysfunction is a very common feature. In advanced stages ascites, hepatomegaly, and jugular venous distension are common. Multi-organ involvement is very common and is seen in close to 50% of AL patients. The kidney, peripheral and autonomic nerves, liver, and gastrointestinal tract are frequent extracardiac sites of disease. Nephrotic syndrome, sensory neuropathy, orthostatic hypotension, hepatomegaly with abnormal liver enzymes, nausea, vomiting, diarrhea, and/or constipation are common clinical manifestations. Soft tissue infiltration is common in AL and gives rise to classic clinical signs of macroglossia, shoulder pad sign, and raccoon eyes (Figs. 34.4 and 34.5). These clinical signs although uncommon (less than 20% of patients), when present, are very specific for AL. Involvement of small- and medium-sized blood vessels by AL can cause symptoms of jaw claudication, and patients can be misdiagnosed as having giant cell arteritis. Occasionally patients with advanced AL present with mucocutaneous bleeding due to failure of hemostasis resulting from altered blood vessel wall integrity and acquired deficiency of circulating coagulation factors, in particular Factor X [24].

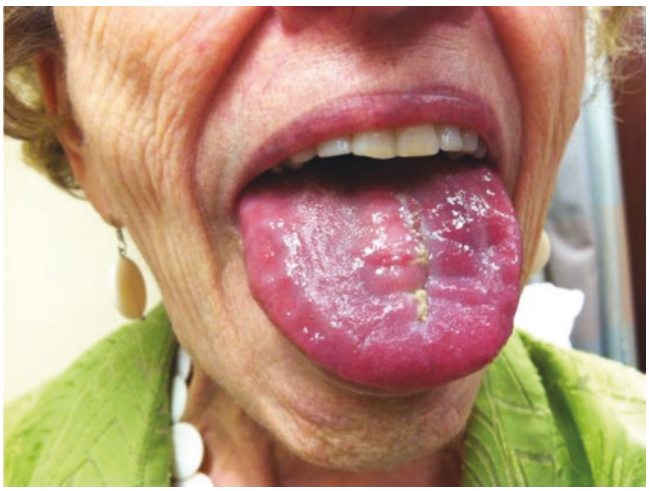

Fig. 34.4 Macroglossia in a patient with advanced multi-system immunoglobulin light chain amyloidosis

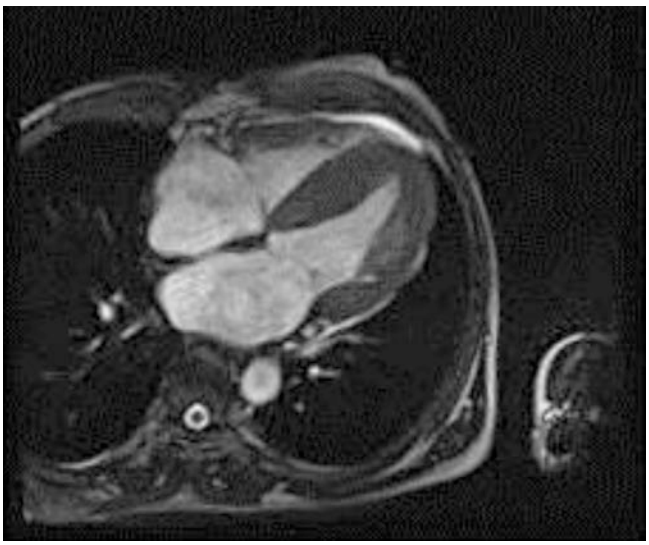

Fig. 34.5 Gadolinium-enhanced MRI of the heart in a patient with advanced AL cardiomyopathy reveals severely thickened interventricular septum. Cardiac disease is the most common cause of mortality in patients with AL

Primary treatment of AL involves treating the underlying plasma cell dyscrasia to eliminate the production of amyloidogenic light chains. This is best achieved by systemic chemotherapy as soon as the diagnosis is established. Systemic therapy improves organ function, quality of life, and survival of patients. The availability of the serum free light chain assay and cardiac biomarkers Troponin T and NT-ProBNP has proved invaluable in monitoring disease response to treatment and determining the prognosis of the patient [25]. Hematological response to treatment precedes an organ response and has been shown to be a strong predictor of not only organ improvement but also overall survival. Patients who achieve a complete hematological remission (CR = normal serum free light chain levels and a normal kappa to lambda ratio and no evidence of clonal plasma cells) have a 66–86% chance of organ improvement and 5-year survival that approaches 70%. Improvement in organ function and survival is also noted in patients who achieve partial hematological remission (PR = at least 50% reduction from baseline in involved free light chain levels) [26].

AHCT is very effective in AL but is associated with a high risk of complications including high treatment-related mortality. Early experience reported mortality in excess of 20% in the USA and more than 40% in a small European series. The availability and application of cardiac biomarkers and the use of stringent selection criteria have significantly improved the transplant- related mortality to less than 3% in experienced centers. At the Mayo Clinic, active NYHA class III or IV heart failure, LVEF <45%, advanced cardiac stage by biomarkers, and more than two organ involvement are considered a contraindication for SCT. Using these stringent criteria, only about 25% of AL patients qualify for SCT at diagnosis [27]. Proteasome inhibitors and the anti-CD38 monoclonal antibody daratumumab have shown remarkable activity in AL and are undergoing active investigations in randomized studies. Like MM, supportive care is important in the management of AL. It involves both managing the cardiac manifestations of the disease and systemic chemotherapy. Management of heart failure in AL is particularly challenging, as these patients cannot tolerate several medications used for routine therapy of heart failure from other causes. Use of ACE inhibitors is particularly difficult as they result in significant hypotension by causing marked reduction in cardiac pre-load. Orthostatic hypotension can become particularly problematic, and patients may benefit from compression stockings and use of the alpha agonist midodrine. Volume overload can be a recurrent and difficult-to-treat problem; salt restriction, loop diuretics, and potassium-sparing agents should be used with strict monitoring of blood pressure and orthostatic responses. Outcomes of relapsed AL are generally poor. The best option for these patients is enrollment in clinical trials whenever possible or the use of the same or non-cross-resistant chemotherapy regimens depending upon the length of initial response prior to disease relapse. Prognosis of AL is highly dependent upon the degree of cardiac involvement, and survival of patients ranges from 3 months or less to in excess of 15 years depending on the degree of cardiac involvement.

Other Plasma Cell Disorders

POEMS (polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, and skin) is a rare type of PCD where transformed plasma cells with low proliferative potential cause systemic manifestations through paraneoplastic phenomenon. The exact pathophysiology of this disorder is not known; however, vascular endothelial growth factor (VEGF) plays a central role in causing fluid overload, papilledema, and possibly neuropathy. The treatment involves the use of antineoplastic therapy to eliminate the plasma cells. IMiDs such as lenalidomide and pomalidomide result in good response. Patients who are candidates for SCT should undergo this modality in a center experienced in dealing with such patients as peri-engraftment syndrome is more common in these patients. Following VEGF levels is an important component of monitoring disease activity and response to treatment.

Light and heavy chain deposition diseases are an uncommon type of PCD where the light chain and heavy chains or their fragments deposit in tissues in non-fibrillary form (unlike AL where the amyloid fibrils from light or heavy chains deposit in tissues) and cause organ damage. Such deposits are most commonly identified as patients undergo workup for unknown kidney disease, and renal biopsy shows fragments of light chains in Bowman's capsule and peritubular areas. Other organs can be involved (liver and heart). Like AL these disorders are best treated with timely recognition and instituting anti-plasma cell therapy to eliminate the production of pathogenic Ig and its components. Outcomes of patients depend on the severity of the organ damage.

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Neoplastic Hematologic Disorders in Children and Adolescents

35

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Abbreviations

ADCs	Antibody-drug conjugates
ALL	Acute lymphoblastic leukemia
ALT	Alanine aminotransferase
AML	Acute myeloid leukemia
ANV	Anticipatory nausea and vomiting
APCR	Activated protein C resistance
AST	Aspartate aminotransferase
ATG	Anti-thymocyte globulin
ATP	Adenosine triphosphate
AUL	Acute unclassified leukemia
BCP-ALL	B cell precursor ALL
BCR	Breakpoint cluster region
BID	Bis in die
BiTE	Bispecific T cell-engaging antibody
BM	Bone marrow
BSA	Bovine serum albumin
BW	Body weight
CALR	Calreticulin
CAR T cells	Chimeric antigen receptor-modified T cells
CD	Cluster of differentiation
CINV	Chemotherapy-induced nausea and vomiting
CLL	Chronic lymphoblastic leukemia
CML	Chronic myeloid leukemia
CMV	Cytomegalovirus
CNS	Central nervous system
CRP	C-reactive protein
CSA	Cyclosporine A
CTL	Cytotoxic T lymphocytes
cyCD	Cytoplasmic CD
d	Day
DIC	Disseminated intravascular coagulation
DNA	Desoxyribonucleic acid
EBV	Epstein-Barr virus
ECG	Electrocardiography
EEG	Electroencephalography
EPO	Erythropoietin
ET	Essential thrombocythemia
FAB	French-American-British classification system
FDA	US Food and Drug Administration
FU	Follow-up
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GvHD	Graft-versus-host disease
GvL	Graft-versus-leukemia effect
Gy	Gray
Hb	Hemoglobin
HbF	Fetal hemoglobin
HHV	Human herpes virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transplantation
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
INR	International normalized ratio
ITP	Idiopathic thrombocytopenic purpura
IV	Intravenous
JAK	Janus kinase
JMML	Juvenile myelomonocytic leukemia
LDH	Lactate dehydrogenase
L-DNR	Liposomal daunorubicin
m^2	Square meter
MDS	Myelodysplastic syndromes
MFD	Matched family donor
mg	Milligram
mL	Milliliter
MMFD	Mismatched family donor
MMUD	Mismatched unrelated donor

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MPD	Myeloproliferative diseases
MPL	Thrombopoietin receptor
MPN	Myeloproliferative neoplasm
MPS	Myeloproliferative syndromes
MRD	Minimal residual disease
MRI	Magnetic resonance imaging
MUD	Matched unrelated donor
NF1	Neurofibromatosis type 1
NK1R	Neurokinin 1 receptor
PAS	Periodic acid-Schiff stain
PCR	Polymerase chain reaction
pCRT	Prophylactic CNS radiotherapy
Ph^1	Philadelphia chromosome
PMF	Primary myelofibrosis
PO	Per os
PTT	Partial thromboplastin time
PV	Polycythemia vera
RA	Refractory anemia
RAEB	RA with excess of blasts
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcriptase PCR
SAA	Severe aplastic anemia
sIg	Surface immunoglobulin
SMN	Secondary malignant neoplasms
TCR	T cell receptor
tCRT	Therapeutic CNS radiotherapy
TdT	Terminal deoxynucleotidyl transferase
TID	Ter in die
TKI	Tyrosine kinase inhibitors
TMP/SMX	Trimethoprim/sulfamethoxazole
TNF-α	Tumor necrosis factor alpha
VZV	Varicella zoster virus
WHO	World Health Organization
µL	Microliter

Cancer in Children and Adolescents

General Information

Leukemia is a malignant disease of the bone marrow, characterized by an uninhibited clonal expansion of hematopoietic progenitor cells suppressing the normal hematopoiesis. Depending on the cell type of the leukemia-initiating cell (tumor stem cell), two forms are distinguished, namely, lymphoblastic and myeloid leukemia. According to the natural progression of the disease, leukemia is further classified as acute or chronic. If not treated, leukemia is inevitably fatal. Pediatric patients without appropriate treatment of acute leukemia will only survive for weeks to a few months. By contrast, children with chronic leukemia may survive for several years. The aim of the treatment is to achieve a remission through the targeted use of cytostatic agents, reaching a condition where leukemia cells are not detectable in the bone marrow and normal hematopoiesis is possible again. Pediatric patients who remain in complete remission for at least 5 consecutive years are considered to be "cured," since relapse of the disease is rarely observed after this period of time.

Epidemiology

Leukemia and myeloproliferative and myelodysplastic diseases are the most prevalent causes of cancer, accounting for about 35% of malignant diseases in children and adolescents. Approximately 1 in 1200 children under the age of 15 years is affected, boys approximately 20% more often than girls. About half of cases are associated with onset at age ≤6 years, and the median age of diagnosis is between 1 and 5 years. In children and adolescents, acute forms of leukemia are prevalent, while in adults chronic forms more commonly occur. In about 80% of cases involving children, acute lymphoblastic leukemia (ALL) is diagnosed and associated with a long-term survival of about 89%, which has continued to increase over time. Acute myeloid leukemia (AML) is much rarer in children, accounting for approximately 16–18% of cases, and it has a much poorer prognosis with a long-term survival of about 71%. Myelodysplastic syndromes (MDS) are only classified as malignant diseases since the beginning of the millennium, and a percentage of those affected by MDS may experience development into an AML. About 4% of cases are chronic myeloid leukemia (CML), juvenile myelomonocytic leukemia (JMML), acute unclassified leukemia (AUL), and occasionally occurring chronic lymphoblastic leukemia (CLL) [1].

Causes of Pediatric Neoplasia

Major influencing factors during the tumorigenesis in leukemia are endogenous genetic factors, but exogenous factors also have an important role. The specific mechanism of the development of leukemia remains mostly unclear. However, it is known that specific factors increase the risk of developing leukemia, including genetic predisposition and exposure to radioactive radiation as well as chemical substances. For some rare forms of leukemia, viruses are involved in the tumorigenesis. For most cases, no specific trigger can be identified for the development of the disease. In the strict sense, there are no hereditary forms of leukemia. However, there is evidence that the risk for leukemia increases for the more malignant diseases that occur in close relatives, whereby this observation strengthens the assumption of a genetic predisposition for leukemia. Furthermore, specific genetic conditions increase the risk of developing leukemia. Thus, people with trisomy 21 (Down syndrome) have a 20-fold higher risk of developing leukemia compared to

healthy subjects [2]. Furthermore, it has been shown that leukemia cells frequently contain chromosomal aberrations. The most frequent aberration is the so-called Philadelphia chromosome (Ph^1), which is present in a very high percentage of CML patients. It develops through a reciprocal translocation between chromosomes 9 and 21. Through this translocation, the ABL1-protooncogene of chromosome 21 is transferred to the breakpoint cluster region (BCR) of chromosome 9, causing the expression of the fusion protein BCR-ABL1, which comprises a constitutive tyrosine kinase activity. This genetic modification is the major cause for the malignant transformation of a healthy hematopoietic cell and the subsequent development of leukemia. These modifications are not hereditary but may occur in the course of one's life [3].

As seen in the course of time after the atomic bombing of Hiroshima and Nagasaki, radioactive radiation significantly promotes the occurrence of acute leukemia. Likewise, several chemical agents such as benzene or other organic solvents as well as insecticides and herbicides may increase the risk of leukemia.

Therapy

Usually, therapy of leukemia comprises repeated phases of induction, consolidation, and re-induction chemotherapy followed by maintenance therapy. The phases of induction, consolidation, and re-induction chemotherapy also are referred to as the intensive phase of the therapy. The total duration of the intensive phase depends on the individual risk factors and the course of treatment of each patient.

Induction Phase

The aim of the induction therapy is maximum destruction of the leukemic cells. Therefore, various cytotoxic or cytostatic agents are combined and in some cases repeatedly administered over several weeks. Through this intensive treatment, the remaining normal hematopoiesis is massively impaired, leading to a protracted hypoplasia or aplasia of the bone marrow. Pediatric and adolescent patients are thus highly susceptible to infection or bleeding and therefore require intensive monitoring, treatment of concomitant complications, and transfusion support with thrombocytes and occasionally granulocytes during this treatment phase.

Consolidation Phase

After achieving a complete remission through the induction therapy, the consolidation therapy follows to destroy residual but morphologically undetectable leukemia cells.

Re-induction Phase

The consolidation phase is followed by the re-induction phase, during which chemotherapeutic treatment is again intensified by polychemotherapy.

Central Nervous System (CNS) Prophylaxis for Hematologic Diseases

Many cytotoxic or cytostatic agents do not or only rarely pass through the blood-brain barrier. When leukemic blasts pass the blood-brain barrier and thus enter the central nervous system (CNS), they may induce a relapse of the disease. In order to prevent these forms of relapse, all pediatric and adolescent patients undergo a so-called CNS prophylaxis, which can be performed in two different ways: by repeated direct administration of cytotoxic agents into the cerebrospinal fluid (CSF) and by lumbar puncture as either a monotherapy, e.g., with methotrexate (ALL); a triple therapy (ALL relapse, AML) with a combination of methotrexate, cytarabine, and prednisolone; or a double therapy with methotrexate/prednisolone or cytarabine/prednisolone (babies <1 year of age with ALL). Dosing is adjusted according to the patient's age [4]. Additionally, CNS radiotherapy can be performed for CNS prophylaxis.

Maintenance Therapy

After achieving complete remission through induction, consolidation, and re-induction chemotherapy, maintenance therapy follows to eliminate residual leukemia cells. Consistent dose adjustment to changing blood counts is essential during maintenance therapy.

Radiotherapy

CNS Radiotherapy During Hematologic Diseases

Based on the observed late effects in most therapy studies with children and adolescents, CNS radiotherapy is successively being replaced by intrathecal mono-, double, or triple chemotherapy [5]. However, for some risk groups, prophylactic or therapeutic CNS radiotherapy still may be necessary.

Prophylactic CNS Radiotherapy

Prophylactic CNS radiotherapy (pCRT) is performed in pediatric patients without proof of leukemic blasts in the CSF but with specific risk factors.

Therapeutic CNS Radiotherapy

Therapeutic CNS radiotherapy (tCRT) is performed in pediatric patients with proof of leukemic blasts in the CNS during the initial treatment or with the occurrence of an isolated or combined CNS relapse. The therapeutic radiation dose in pediatric patients >1 year of age and <2 years of age is 12 Gray (Gy). Patients ≥2 years of age are treated with 18 Gy.

Side Effects of CNS Radiotherapy

The risk of side effects during radiotherapy is relatively low but differs individually. Some patients experience headache with nausea and vomiting. Temporary medication with dexamethasone can be used at a dosage of 10 mg/m^2 body surface area in three individual doses (TID) and a maximum dose of 3×8 mg per day. Rashes with dry scaling can occur in the radiated area, depending on the administered doses and radiation technology, as well as varying individually. Especially during spring and summer months, a direct exposure of the radiated area should be avoided. Furthermore, hair loss frequently occurs at the area where radiation meets the hair follicles. Both side effects usually decline after finishing the radiotherapy. Permanent skin changes, skin browning, or thinning and hardening of the subcutaneous tissue occur rarely. The side effects that occur during or shortly after radiotherapy are separated from late effects that may occur months to years after the end of the radiotherapy.

Long-Term Effects of CNS Radiotherapy

With increasing cure rates, the risk of long-term effects of radiotherapy gains increasing focus. The risk of later acquiring a secondary malignant disease is even increased with concomitant chemotherapy during CNS radiation. While secondary malignant neoplasms (SMN) normally occur within the first 5 years after chemotherapy, radiotherapy-related secondary tumors may develop after this period of time [5–10]. Cranial irradiation can impair the production of growth and other hypophyseal hormones that partially regulate the production of the sex hormones. An impairment of this system can result in growth restrictions or developmental disorders, e.g., in the form of a delayed or missed puberty. Regular monitoring of female and male sex hormones enables a timely substitution, if necessary. The cranial irradiation may also impair intellectual abilities such as concentration, attention, retentiveness, memory, and learning capability. However, most patients are able to maintain a satisfying quality of life and performance in daily life by individual compensation mechanisms and specific support [11]. If salivary glands were exposed to the radiation field, the common xerostomia can be treated with additional fluid or by artificial saliva.

Supportive Care

The supportive care aims to reduce or prevent major side effects of intensive polychemotherapy. This care program includes therapeutic efforts for mucositis, nausea, and vomiting as well as prevention and treatment of viral, fungal, and bacterial infection.

Antiemetic Prophylaxis and Therapy

The majority of administered chemotherapeutic agents potentially cause chemotherapy-induced nausea and vomiting (CINV). A timely start before the administration of emetic chemotherapy and sufficient duration (usually 3–4 days) of antiemetic prophylaxis are crucial for the treatment efficacy. Antiemetic prophylaxis is adapted to the emetogenic potential of the administered chemotherapeutic drugs. After the start of emetic chemotherapy, CINV may occur within the first 24 h after application (acute phase) or between 25 and 120 h after application (delayed CINV phase). Delayed CINV is distinctly more difficult to control than acute CINV. The most important agents for antiemetic prophylaxis include 5-HT3 receptor antagonists (ondansetron, granisetron), NK1 receptor antagonists (aprepitant, fosaprepitant), and the halogenated glucocorticoid dexamethasone. H1 receptor antagonist (dimenhydrinate), phenothiazine (levomepromazine), and dopamine antagonists (metoclopramide) complement this prophylaxis regimen as rescue medication.

CINV must be differentiated from anticipatory nausea and vomiting (anticipatory nausea and vomiting (ANV); prophylaxis with lorazepam). The most emetogenic factor is the chemotherapy, while the administered doses, form of application, and combination and type of the chemotherapy play an important role.

Prophylaxis and Therapy of Mucositis

Mucositis is a common complication during high-dose methotrexate therapy, high-dose chemotherapy, or therapy with Adriamycin, busulfan, bleomycin, cytarabine, dactinomycin, daunorubicin, etoposide, idarubicin, or melphalan. With mucositis, the inflammation and ulceration are worsened by bleeding, superinfections (e.g., with herpes viruses), fungal infections (e.g., *Candida* spp., *Aspergillus* spp.), or bacteria. The chemotherapy causes a disruption of the regenerative processes of the mucous membranes. Consequently, toxicity commonly occurs 2–7 days after chemotherapy in the form of isolated or connected lesions on the mouth base, the buccal mucosa, the tongue, or the soft palate. The lesions are treated with disinfecting mouthwash (with or without a local anesthetic) and an appropriate pain therapy (e.g., with mor-

phine derivatives). Cold drinks, lollipops, or ice cubes are preferred foods. The indirect mucosal toxicity is caused by the bone marrow depression after chemotherapy and can be treated with granulocyte colony-stimulating factor (G-CSF). Depending on the intensity and localization of the mucositis, damage to the gastrointestinal mucosa occurs, causing a malabsorption and increasing the risk of bacterial infection or sepsis [12, 13].

Prophylaxis of Infectious Complications

The most important measures of the pharmacological prophylaxis include protection against the *Pneumocystis jirovecii* pneumonia (PJP) in all patients, an antifungal prophylaxis for high-risk patients, different antiviral prophylaxis regimen, and the administration of hematopoietic growth factors (e.g., G-CSF). Further additional prophylactic antibiotic measures are not recommended.

Pneumocystis jirovecii Prophylaxis

During and up to 1 month after finishing chemotherapy, a *Pneumocystis jirovecii* prophylaxis with trimethoprim/sulfamethoxazole (TMP/SMX) is recommended. If intolerance toward these agents exists, inhalation with pentamidine is an alternative. Moreover, chemoprophylaxis with 5 mg per kg body weight (BW) oral TMP in two separate doses per day (BID) on 2–3 days per week may be used.

Antifungal Prophylaxis

In high-risk patients with (anticipated) prolonged granulocytopenia of more than 1 week (e.g., high-risk ALL, AML), pharmacologic prophylaxis can reduce the risk of invasive *Aspergillus* and *Candida* infections. Antifungal prophylaxis includes intravenous liposomal amphotericin B at 1 or 3 mg/kg body weight per day. During aplasia between two chemotherapy courses, an antifungal prophylaxis with itraconazole or voriconazole is recommended.

Antiviral Prophylaxis

During the reduced lymphocyte activity during chemotherapy, pediatric patients may experience life-threatening complications through virus infections, mainly from the herpes group, especially varicella zoster virus (VZV). Besides prophylaxis, *postexposure* prophylaxis is potentially used. During pronounced stomatitis after therapy with methotrexate, reactivation of a herpes infection has to be considered. In the case of recurrent herpes simplex infection, a secondary prophylaxis with acyclovir can be considered.

Varicella Zoster Virus Postexposure Prophylaxis

A passive immune prophylaxis with intravenous varicella zoster (VZ) immunoglobulins (1 mL per kg body weight) is recommended for VZV-seronegative and immunosuppressed patients within 24 to a maximum 96 h after contact, as well as a chemoprophylaxis with oral acyclovir 60–80 mg/d divided into 3–4 doses for 14 days from the moment of exposure.

Substitution of Blood Components

The indication for blood transfusion has to be determined very carefully, adhering to strict guidelines. Each transfusion contains the risk of a virus transmission (hepatitis B virus, hepatitis C virus, cytomegalovirus, human immunodeficiency virus) and other severe transfusion-related adverse events.

In highly immunosuppressed patients, there is a high risk of a graft-versus-host reaction against transfused viable donor lymphocytes contained within the blood product. Therefore, the use of external irradiation of transfusion products with 25–30 Gy is recommended [14]. Another transfusion-associated adverse event is febrile transfusion reactions that occur due to the sensitization against human leukocyte antigen (HLA) antigens. The risk of this adverse reaction is significantly reduced by leukocyte depletion of erythrocyte concentrates. The indication for transfusion of leukocyte-depleted erythrocyte concentrates depends on the patient's clinical status and the anticipated regeneration of his/her hematopoiesis. The transfusion-related increase of hemoglobin can potentially intensify a leukostasis situation. If possible, a chemotherapeutic cytoreduction should be undertaken before transfusion. A transfusion should be considered at hemoglobin values ≤6 g/dLl or at hemoglobin values between 6 and 8 g/dL and clinical signs of an anemia, such as tachycardia, fatigue, or headache. An indication for platelet concentrate transfusion is manifest bleeding (macroscopic hematuria, gastrointestinal bleeding, suspected intracranial bleeding). A relative indication is given by an increase in or very pronounced skin hemorrhages such as petechiae, hematoma, or oozing/bleeding. Clinically relevant bleeding is expected at thrombocyte counts <10.000/microliter (μL).

Acute Complications During Chemotherapy

Metabolic Complications

Tumor Lysis Syndrome

The tumor lysis syndrome is frequently observed in patients with a high initial tumor burden of leukocyte count. It is accompanied with hyperuricemia, hyperphosphatemia, hyperkalemia, and acute kidney failure. These laboratory chemical changes are caused by a massive and acute cell death in the course of the initial intensive chemotherapy. *Lactic acidosis*, precipitation of uric acid crystals in the collecting duct system, and hypocalcemia are additional risk factors. The hyperkalemia following a massive release of intracellular potassium may cause a sudden cardiac death.

The most important therapeutic intervention during tumor lysis syndrome is thus sufficient hydration and the administration of an isotonic-balanced electrolyte solution of at least 3 L/m^2 per day depending on the cell death. An alkalization of the urine is not necessary. An enzymatic induction of the uric acid metabolism by cleavage of the uric acid by uratoxidase (rasburicase) at a dosage of 0.1–0.2 mg/kg body weight is recommended. This treatment can be repeated daily. Furthermore, allopurinol therapy has to be initiated as early as possible to prevent a further production of uric acid. At very high leukocyte counts of >250,000/μL, a therapeutic leukapheresis can be indicated [15]. Thus, regular blood testing is recommended including whole blood count, electrolytes (potassium, sodium, and calcium), uric acid, urea, creatinine, and blood coagulation factors. Depending on the general condition of the patient, blood testing should be performed every 4, 8, 12, or 24 h. The hyperkalemia that is intensified by renal insufficiency should be treated with potassium absorbents or a glucose-insulin therapy. In some cases, a hemodialysis is necessary.

In order to prevent a tumor lysis syndrome, a patient-adapted, dose-reduced chemotherapy order or low-dose corticosteroids can be administered initially. A normal-dose, more aggressive, and guideline-conform chemotherapy can directly follow after a few days.

Organ-Specific Complications

Peripheral Neuropathy

The mixed sensorimotor neuropathy after vinca alkaloids (vincristine, vinblastine, vindesine) is localized in the lower extremities and progresses proximally. Furthermore, weakened muscle reflexes, paresthesias of the distal extremities, muscle weakness of the foot or hand flexors, mandibular pain, and paralysis of the brain and larynx nerves can occur. If the autonomous nerve system is affected, symptoms such as obstipation, paralytic ileus, urinary retention, hypertension, and impotence are observed. The neurotoxicity is presumably caused by an interaction of vincristine and the microtubules blocking the axonal transport and inducing an axonal degeneration [16]. During severe pain, an anticonvulsive therapy with pregabalin or gabapentin is indicated.

Coagulation Disorders

Disseminated Intravascular Coagulation (DIC)

Disseminated intravascular coagulation (DIC) is a coagulopathy that is caused by an intravascular activation of the blood coagulation system and frequently occurs in the course of sepsis, primarily in pediatric patients with leukemia (see Chap. 13). The activated coagulation leads to an increased consumption of plasma coagulation factors and thrombocytes, causing a subsequent lack of these components. The release of procoagulant substances from leukemic cells further supports this process, inducing a hemorrhagic diathesis. Diagnostic markers of acute DIC as seen in leukemic patients include an increased prothrombin time, an increased activated partial thromboplastin time (aPTT), an increased thrombin time, reduced fibrinogen plasma concentrations, increased levels of fibrin degradation products in the plasma, and the obligatory thrombocytopenia. The finding of three or more of these parameters prolonged in the leukemic patient is DIC until proven otherwise.

Thrombophilia

Owing to the changed characteristics of blood cells, blood plasma, blood circulation, or vascular walls, thrombophilia is characterized by an increased risk of thrombosis. Genetic risk factors for thrombophilia are activated protein C APC resistance (APCR; factor V Leiden mutation), prothrombin mutation G20210A, antithrombin deficiency, protein C deficiency, and protein S deficiency. Diagnosis of these genetic mutations and thrombophilia is essential prior to initiating chemotherapy. Patients with risk factors for thrombophilia and a central venous catheter should receive a heparin prophylaxis as long as thrombocyte counts are >50,000/μL to prevent catheter thrombosis, another nidus for infection.

Targeted Therapy

Table 35.1 provides examples of new, targeted therapy strategies for hematologic neoplasms.

Tyrosine Kinase Inhibitors (TKI)

Tyrosine kinase inhibitors (TKI) belong among the most important pharmacologic developments that are licensed for

Table 35.1 Examples of new targeted therapy strategies for hematologic neoplasms

Substance	Trade name	Mechanism of action/substance class
Imatinib	Glivec	Selective TKI
Dasatinib	Sprycel	TKI during imatinib resistance
Nilotinib	Tasigna	TKI during imatinib resistance
Rituximab	Mabthera	Chimeric anti-CD20 antibody
Ibritumomab tiuxetan	Zevalin	Yttrium-coupled anti-CD20 antibody
Alemtuzumab	MabCampath	Humanized anti-CD52 antibody
Gemtuzumab ozogamicin	Mylotarg	Immunotoxin: anti-CD33 antibody with calicheamicin
Thalidomide	Contergan	Immunomodulating agent
Ponatinib	Iclusig	Selective TKI

hemato-oncologic indications. They are relatively specific to a class of cellular-signaling proteins that have a key role in the tumorigenesis when constitutively activated: the tyrosine kinases [17, 18]. TKI can be administered orally and are used as single-agent therapies or in combination with chemotherapy or other targeted therapies. The mechanism of action of all licensed low-molecular TKI is based on the inhibition of the catalytic activity of the target tyrosine kinase by blocking either the nucleotide binding [adenosine triphosphate (ATP) binding site] or the substrate binding (substrate binding site) [19]. Most currently available TKI are ATP-competitive molecules, which means that by blocking the ATP-binding site of the enzyme its kinase activity is inhibited [20]. Some TKI such as imatinib additionally comprise an inhibitory effect by conformational changes fixating the enzyme in an inactive form (semi-competitive inhibition) [21]. The majority of clinically useful TKI are targeted against tyrosine kinases located in the membrane (receptor tyrosine kinases; RTK) that mediate extracellular signals. The high specificity of TKI results in a low range of biological activity, with exception of ponatinib. Thus, in some cases, molecular testing needs to be conducted prior to the use of TKI to ensure that the patient is able to benefit from the treatment.

BCR-ABL Inhibitors

For the therapeutic inhibition of the constitutively active BCR-ABL kinase in the Philadelphia chromosome-positive CML (Ph+), five different substances are currently licensed: imatinib (Glivec®), nilotinib (Tasigna®), dasatinib (Sprycel®), ponatinib (Iclusig®), and bosutinib (Bosulif®). All five TKI inhibit additional kinases besides the BCR-ABL. Imatinib inhibits the proliferation and induces the differentiation of leukemia cells and the apoptosis. Not least due to the good tolerability, imatinib has become the first-line therapy. The second-generation TKI dasatinib and nilotinib are used as first- and second-line therapy [22, 23]. The third-generation TKI bosutinib and ponatinib are used as second- and third-line therapy [24, 25]. Ponatinib – which is licensed for the use of Ph+ ALL – is especially used during T315I mutations of BCR-ABL or resistance against other TKI [26].

Jak1/2 Inhibitors

In 2012, ruxolitinib (Jakavi®) was the first licensed inhibitor of the Janus kinases Jak1 and Jak2. This discovery enabled the first targeted therapy for myelofibrosis, which typically includes a constitutive activation of the Jak/STAT signaling pathway. In the approval studies, ruxolitinib significantly improved the disease-related symptoms when compared to a placebo. Furthermore, a long-lasting positive effect regarding the spleen size and the quality of life was observed under ruxolitinib [27, 28]. Ruxolitinib is recommended as the standard therapy against myelofibrosis.

Side Effects

Imatinib (Glivec®), nilotinib (Tasigna®), dasatinib (Sprycel®), ponatinib (Iclusig®), and bosutinib (Bosulif®) comprise an inherent marrow suppression that may necessitate dose reductions or an interruption of the therapy. During therapy with these substances, fluid retention, diarrhea, exanthema, and myalgia occur [29]. The substance-specific side effects of dasatinib include pleural effusion or pulmonary hypertension [30]. During therapy with nilotinib hyperglycemia, increases of amylase and lipase as well as pancreatitis frequently occur. Under bosutinib therapy, mainly gastrointestinal side effects are observed.

To date, TKI therapy has enabled a relatively targeted therapy that is normally associated with fewer side effects. Thus, hemato-oncologic therapy has gained another pillar that has replaced chemotherapy for several indications. At present, continuous TKI therapy achieves at best a disease control at the level of a minimal residual disease [31].

Monoclonal Antibodies

Monoclonal antibodies are antibodies that are produced by cell lines that derive from a single B-lymphocyte and that are targeted against a single epitope. Monoclonal antibodies have an important role in diagnostics and research because they can bind a variety of molecules with a high specificity. Several newly developed immunotherapy products including antibody-drug conjugates, bispecific T cell-engaging antibodies (BiTE), or chimeric antigen receptor (CAR)-modified T cells have shown promising results and may significantly improve the outcome of relapsed ALL. For example, blinatumomab is a recently FDA-approved (US Food and Drug Administration) CD3/CD19-bispecific T cell-engaging antibody for the treatment of Ph^{1}-negative relapsed or refractory ALL [32]. It binds the surface B cell-specific surface protein CD19 (also B-lineage leukemia cells and lymphoma cells) and the ε subunit of CD3, which is a part of the T cell receptor complex on mature T cells.

By binding the target proteins both CD3 and CD19, these BiTE antibodies cross-link a malignant B cell to a T cell. The T cell is thus activated and initiates the production and release of cytotoxic proteins such as the pore-forming protein, which induces the apoptosis. Subsequently, the malignant B cell is destroyed. Blinatumomab is administered intravenously and exhibits an average half-life of approximately 2 h, with likely linear pharmacokinetics. A steady-state concentration is reached within 1 day. The degradation of blinatumomab is most likely enzymatic. Antileukemic activity of blinatumomab with complete minimal residual disease (MRD) response has been shown in pediatric patients with relapsed or refractory BCP-ALL, indicating an important treatment option of blinatumomab in these patients [33].

Antibody-drug conjugates (ADCs) are tripartite molecules consisting of a monoclonal antibody, a covalent linker, and a cytotoxic payload. ADC development has aimed to target the specificity inherent in antigen-antibody interactions to deliver potent cytotoxins preferentially to tumor cells and maximize antitumor activity and minimize off-target toxicity. The ADC inotuzumab ozogamicin, brentuximab vedotin, and gemtuzumab ozogamicin have been approved for hematologic malignancies. Inotuzumab ozogamicin is an antibody-drug conjugate composed of a monoclonal antibody targeting CD22, a cell surface antigen expressed on cancer cells in almost all B cell precursor ALL patients. Hepatotoxicity, including fatal and life-threatening VOD, occurred in patients during or following treatment with inotuzumab or following subsequent HSCT [34, 35].

CAR T Cell Therapy

Relapsed or refractory ALL is still associated with an extremely poor prognosis, especially in pediatric patients, being the major cause for death from childhood cancer. Engineered T cell therapy represents a new treatment option in these patients [36–38]. Novel CAR and modified T cells targeting CD19 have proven effective in patients with ALL, CLL, and B cell lymphoma in case series [39]. CAR are genetically engineered (lentiviral transduction) receptors expressed on T cells that link anti-CD19 single chain Fv domains to intracellular T cell-signaling domains of the T cell receptor, redirecting cytotoxic T lymphocytes (CTL) to cells that express this antigen [40]. However, this T cell activation may cause a mild to life-threatening cytokine release syndrome (CRS). After infusion, CRS is initiated by a massive T cell activation leading to the release of large amounts of cytokines and clinically manifests with high-grade fever and cardiovascular complications. These complications are currently managed conservatively, cytokine-directed (e.g., anti-IL6R antibody tocilizumab) and/or with steroids. Neurotoxicity in the form of aphasia, tremors, somnolence, and encephalopathy occurs in up to 39% of the patients. The specific mechanism of this CAR T cell-triggered side effect has not been clarified yet. Neurotoxicity is managed conservatively and if necessary with steroids that rapidly cross the blood-brain barrier [41].

Tisagenlecleucel (B-ALL) 1 and axicabtagene ciloleucel (non-Hodgkin's lymphoma) are the first FDA-approved (for adults) CAR T cell therapies with high remission rates but also very high costs [42].

Acute Lymphoblastic Leukemia (ALL)

Definition

ALL is a malignant disease of the hematopoietic system. It develops in the bone marrow and is generally associated with an overproduction of immature progenitors of lymphocytes, the so-called lymphoblasts. These blasts increase rapidly and uncontrollably and thereby suppress normal hematopoiesis, relevantly reducing the production of healthy leukocytes, erythrocytes, and thrombocytes. Morphological, immunological (Tables 35.2 and 35.3), cytogenetic (Tables 35.4 and 35.5), biochemical, and molecular genetic factors characterize the different ALL subtypes, determining their individual treatment response (Tables 35.2, 35.3, 35.4, and 35.5).

Prevalence

ALL accounts for approximately 80% of pediatric leukemia and is thus the most common form of leukemia in children

Table 35.2 B cell leukemia

	TdT	HLA-DR	CD19	CyCD22	CD24	CD10	CD20	CylgM	sig
Pro-B cell ALL	+	+	+	(+)	(+)	–	–	–	–
Common ALL	+	+	+	+	+	+	(+)	–	–
Pre-B cell ALL	+	+	+	+	+	+	(+)	+	–
B-ALL	(+)	+	+			(+)	+	–	+

CD cluster of differentiation, *cyCD* cytoplasmic CD, *IgM* immunoglobulin M, *HLA-DR* human leukocyte antigen DR, *sIg* surface immunoglobulin, *TdT* terminal deoxynucleotidyl transferase

Table 35.3 T cell leukemia

	TdT	HLA-DR	CD7	CyCD3	CD5	CD10	CD1	CD2	CD3	CD4	CD8
Pro-T cell ALL	+	(+)	+	+	(+)	(+)	–	–	–	–	–
Pre-T cell ALL	+	(+)	+	+	+	(+)	–	+	–	(+)	(+)
Cortical T-ALL	+	+	+	+	+	(+)	+	+	(+)	(+)	(+)
Mature T-ALL	(+)	+	+	+	+	–	–	+	+	+	+

CD cluster of differentiation, *cyCD* cytoplasmic CD, *HLA-DR* human leukocyte antigen DR, *TdT* terminal deoxynucleotidyl transferase

Table 35.4 Percentage distributions of ploidy in ALL

	Number of chromosomes	Percentage of all cases [%]	Prognosis
Hypodiploid	41–45	6	Unfavorable
Pseudodiploid	46	41.5	Individually different
Hyperdiploid	47–50	15.5	Favorable
Hyperdiploid	>50	27.0	
Normal	46	8.0	

Table 35.5 Structural chromosomal aberrations in ALL

Immune phenotype/ translocation	Oncogene/hybrid fusion gene	Characterization of ALL
t(12;21) precursor B/T cell ALL	TEL-AML-1	25% favorable prognosis
t(9;22) = Philadelphia chromosome	BCR-ABL	3–5% unfavorable prognosis
t(1;19)	TCF3_PBXI	Babies: 25% with mediocre prognosis; often high leukocyte counts
t(4;19;11), (11q23)	AF4-MLL	Mostly babies with poor prognosis; 3% in other ALL types
T cell ALL (11;14)	LMO1	Mostly male patients, extramedullary ALL
B cell ALL t(8;14), t(8;22)	MYC-IGH fusion	Mostly male patients; L3 morphology; with intensive therapy usually good prognosis

and adolescents. Children between 1 and 5 years of age are most frequently affected. Apart from early infancy – in which leukemia only rarely occurs – boys are affected more frequently than girls by a ratio of approximately 1.2: 1. The incidence concerns about 38 per 1 million children per year. The median age at diagnosis of a T-ALL is >8 years. T-ALL affects boys more often than girls by a ratio of 4: 1.

Pathogenesis

ALL is characterized by an uncontrolled proliferation of early lymphatic progenitor cells in the bone marrow, whose differentiation is blocked on a specific differentiation stage. The lymphatic blasts of a patient generally comprise individual and specific genetic markers. These so-called clonal markers indicate that the ALL is derived from a malignantly transformed lymphatic stem cell. This transformation takes place at different stages of the lymphatic cell differentiation. Thus, the leukemia cells of the ALL comprise different phenotypic characteristics such as constellations of surface markers that also correlate to the differentiation stage and the clinical manifestation of the disease.

Sometimes there is proof of cytogenetic aberration, which also is characteristic for specific phenotypical and clinical manifestations and can have a prognostic value. Furthermore, they point to genes that are linked to the pathogenesis of the disease. These specific genes or their products are factors involved in signal transductions, regulation of the transcription, cell cycle control, and/or regulation of the apoptosis. These genetic aberrations can thus have complex consequences for the expression of downstream genes and depending on regulatory mechanisms. Additionally, it is assumed that several genetic aberrations are necessary to induce a malignant transformation of a lymphatic progenitor cell. As a result, disturbances of the cell differentiation, a gain of proliferative functions, or a loss of mechanisms that lead to apoptosis occurs. Ultimately, these changes result in a survival advantage of the malignant clone and a differentiation block at a specific stage, analogous to the normal lymphatic progenitor cell. The most important pathogenic and prognostic importance of a single aberration is the translocation t(9;22) (Philadelphia chromosome), which leads to the production of the BCR-ABL fusion protein. This protein has an aberrant tyrosine kinase activity that is causative for the development of a Ph/BCR-ABL-positive ALL [43].

Prognosis

About 80–85% of patients have an event-free survival during the initial 10 years after diagnosis of ALL. Relapse occurs in about 15–20% of the children during maintenance therapy or within the first 6–18 months after discontinuation of the therapy. After 5 years, the relapse risk is very low. An early relapse within the first 6 months after ending the intensive therapy phase is unfavorable. The prognosis of a late relapse varies individually.

Diagnosis

ALL is diagnosed by blood tests such as complete blood count, serum liver and kidney function panels, cell decomposition parameters (e.g., lactate dehydrogenase [LDH[), urea, potassium, phosphate, and blood chemistry. These parameters can provide important information about the specific cell counts and how well the organs are functioning.

The blood cells are viewed under a microscope to check for abnormal shapes or sizes. A bone marrow aspiration is used to detect and determine relative numbers of lymphatic blasts in the bone marrow. A lumbar puncture is used to analyze the CSF to identify an infiltration of the CNS with lymphatic blasts.

Typing

Cytology

ALL is diagnosed by bone marrow puncture in combination with a whole blood count. By definition, the percentage of blasts of nucleated cells in the bone marrow has to be >25% to diagnose an ALL. During hyperleukocytosis, proof of lymphoblasts in the peripheral blood is sufficient for an ALL diagnosis.

Morphology

Lymphoblasts of the B and T cell line usually have an even, unstructured surface, partially with nucleoli in the nucleus, a sharp defined rim, and little cytoplasm. The cytoplasm is less and homogenously stained blue (Figs. 35.1, 35.2, 35.3, and 35.4).

Cytochemistry

In combination with the cytomorphology, the cytochemistry almost always enables a reliable differentiation of an ALL and an AML. Diagnostic procedures include a variety of chemical stains. The periodic acid-Schiff stain (PAS) detects glycogen or glycogen-containing substances. The acid phosphatase stains about 90% of the T lymphocytes. The peroxidase stains cytoplasmic granules of the myeloblasts. The Sudan Black B stain is used for the detection of primary granules of immature myeloid cells. The nonspecific esterase staining shows an intensive staining of monocytic or histiocytic elements.

Immune Phenotyping

Immune phenotyping is the most important classifying method in the diagnosis of leukemia. It is based on detecting

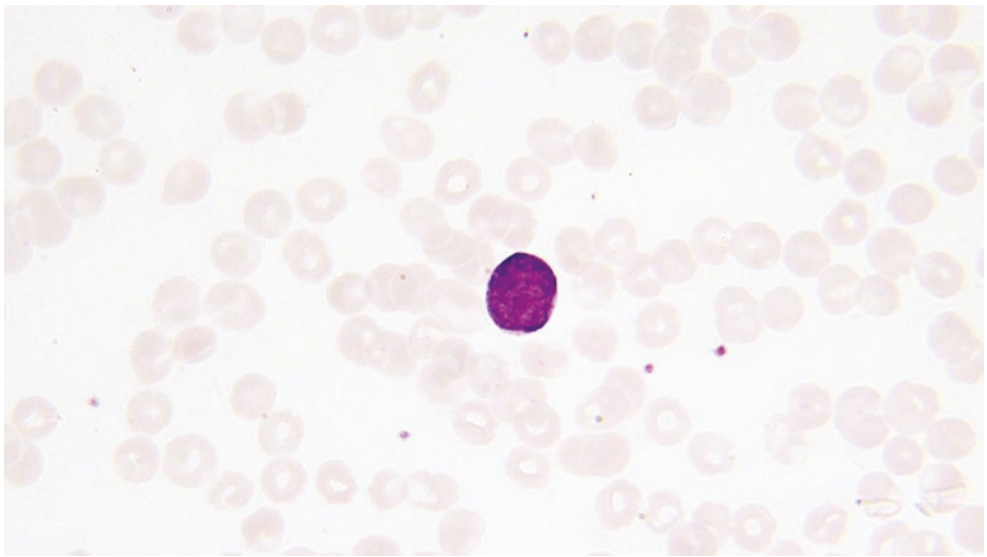

Fig. 35.1 Acute lymphatic leukemia of the B cell line (B-ALL). The blast shows typical characteristics: small with thin or no cytoplasmic seam, a sharp defined rim, and a smooth (immature) nucleic surface, sometimes nucleoli

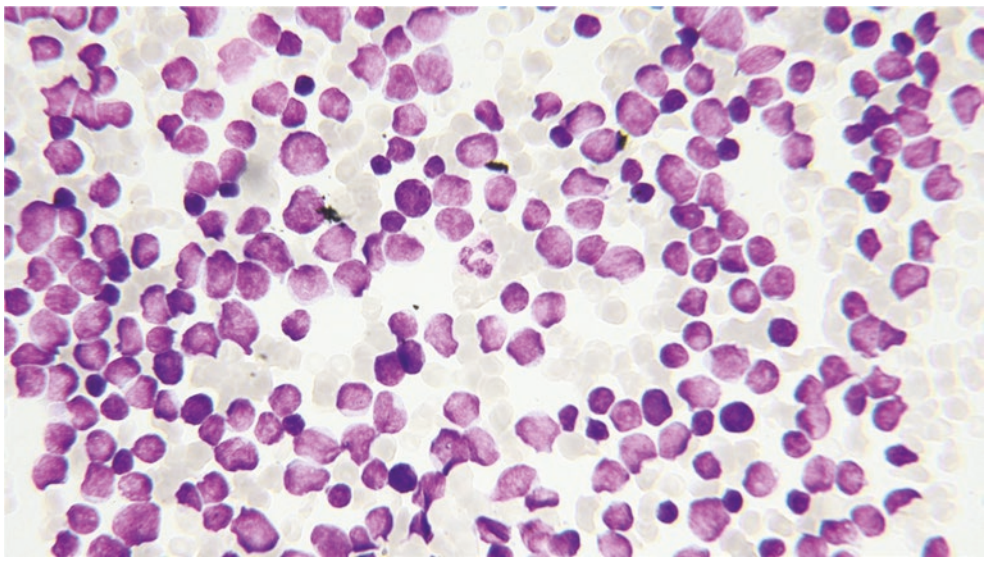

Fig. 35.2 Acute lymphatic leukemia of the B cell line (B-ALL). Many blasts with typical characteristics. They are usually small with thin or no cytoplasmic seam, a sharp defined rim, and a smooth (immature) nucleic surface, sometimes nucleoli

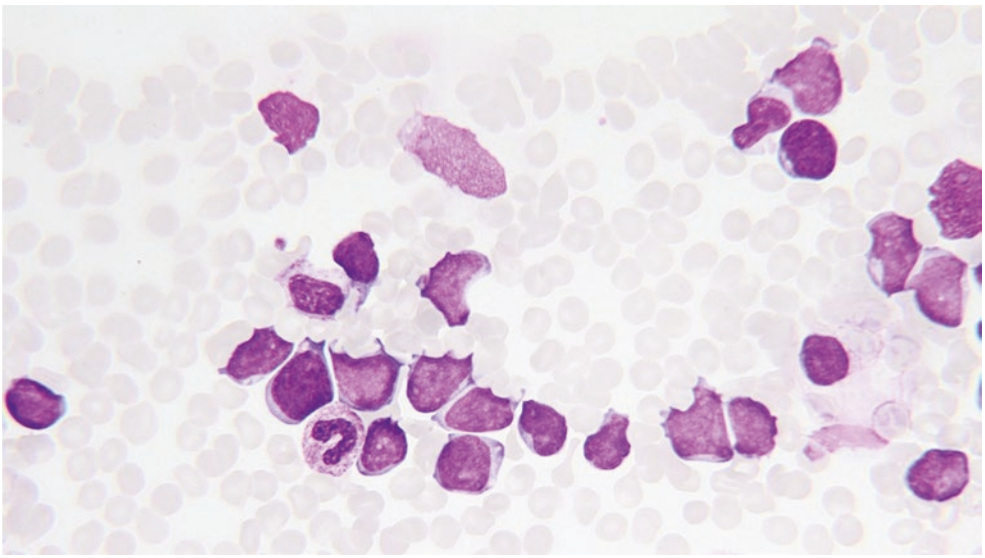

Fig. 35.3 Acute lymphatic leukemia of the T cell line (T-ALL). Blasts are small with thin or no cytoplasmic seam, a sharp defined rim, and a smooth (immature) nucleic surface, sometimes nucleoli, sometimes with a cytoplasmic tail

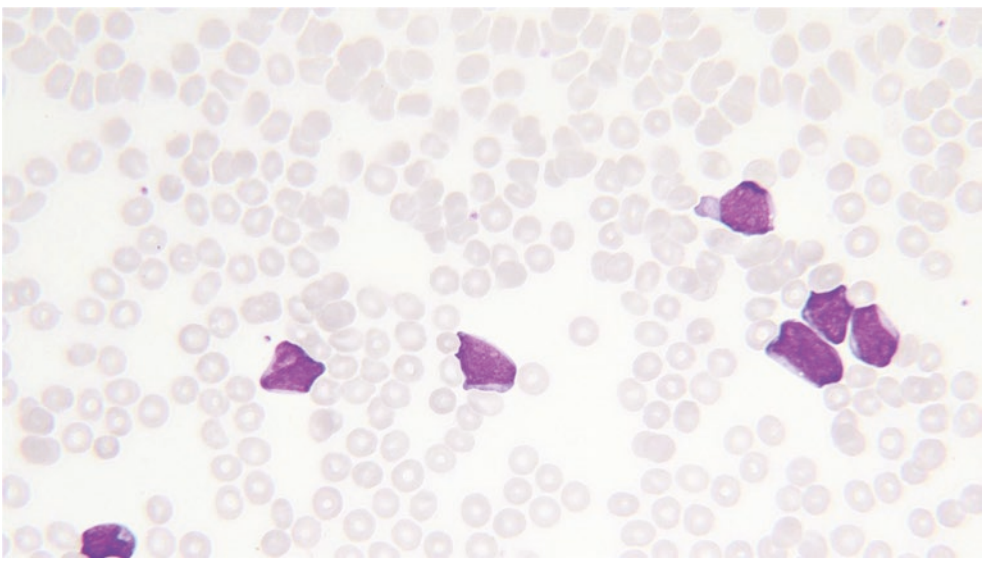

Fig. 35.4 Acute lymphatic leukemia of the T cell line (T-ALL). Blasts are small with thin or no cytoplasmic seam, a sharp defined rim, and a smooth (immature) nucleic surface, sometimes nucleoli, sometimes with a cytoplasmic tail

specific differentiation antigens that are either localized on the cell surface or intracellularly and are visualized by fluorescence-labeled monoclonal antibodies. These different antigens are internationally referred to as cluster of differentiation (CD) antigens. In general, the immune phenotyping enables a safe differentiation of acute lymphoblastic or non-lymphoblastic leukemia and the subclassification of lymphoblastic leukemia according to the specific cell lines (Tables 35.2 and 35.3).

Clinical Symptoms

Bone Marrow Involvement

In general by anamnesis, we recognize severe bone marrow disorders within a couple to several weeks by signs of a pancytopenia (pale skin and/or fatigue), neutropenia with fever, thrombocytopenia with hematoma, petechiae, and more rarely mucosal bleeding as a sign for bleeding and signs for organ infiltration with hepatomegaly, splenomegaly, swollen lymph nodes, or bone pain. Occasionally, headache, signs of meningism, and paresis of the cranial nerves (CNS infiltration) occur. Painless swelling of the testicles, tachypnea, dyspnea, and/or a superior cava syndrome is sometimes observed with mediastinal tumors. As a differential diagnosis, viral infections (e.g., Epstein-Barr virus), severe aplastic anemia (SAA), MDS, idiopathic thrombocytopenic purpura (ITP), rheumatic diseases, or an infiltration of the bone marrow by a different malignant disease needs to be excluded.

CNS Involvement

A CNS involvement occurs in about 4% of cases, usually during a T cell ALL. The symptoms are caused by increased intracranial pressure and include nausea and vomiting, head-

ache, fatigue, seizures, coma, visual disorders, hemi- and paraparesis, paresis of the cranial nerves, hypothalamic syndrome with hyperphagia, or diabetes insipidus.

Testicular Involvement

Testicular involvements clinically manifest as a painless swelling of the testicles. Sonographic examinations and – if necessary – biopsies should be carried out.

Clinical and Laboratory: Chemical Diagnosis

Bone Marrow Involvement

The following are the clinical and laboratory examinations performed for the diagnosis of a potential bone marrow involvement:

- Blood testing with whole blood counts, reticulocyte counts, and a peripheral blood smear; electrolytes (sodium, potassium, chloride, magnesium), inorganic phosphate, liver and kidney parameters, uric acid, C-reactive protein (CRP), LDH, total protein, albumin; coagulation parameters including Quick (PT), international normalized ratio (INR), aPTT, fibrinogen, antithrombin III; and blood grouping, if necessary crossmatch testing for a leukocyte-depleted erythrocyte concentrate (when acute bleeding signs occur during thrombocytopenia, thrombocytes should be given immediately).
- A bone marrow aspirate and biopsy should be performed when two of the three blood cell lines (leukocytes, erythrocytes, and/or thrombocytes) are pathologically changed. The diagnostic studies on the bone marrow include the cytomorphology, immune phenotyping, cytogenetics, and molecular genetics, establishing an MRD marker.
- Exclude a mediastinal tumor – which often occurs with a T cell ALL – and pneumonic infiltrates; a chest X-ray in two planes should be carried out.
- Hepatosplenomegaly, kidney infiltrates or lymph node involvement, exclusion of a local relapse, and painless testicle swelling are assessed by sonographic examinations and testicle biopsies, if necessary.
- Echocardiography and EKG are necessary.

After clinical status assessment and initial leukocyte counts, if necessary:

- Electroencephalography (EEG) and cranial magnetic resonance imaging (MRI) with contrast media.

At diagnosis of BM involvement:

- Virus titers of measles, mumps, rubella, varicella zoster virus, herpes simplex virus, Epstein-Barr virus, cytomegalovirus, human herpes virus 6, parvovirus B19, human immunodeficiency virus 1 and 2, hepatitis A virus, hepatitis B virus, and hepatitis C virus.
- Thrombophilia screening including APCR or factor V Leiden mutation, prothrombin mutation G20210A, antithrombin III, anticardiolipin and anti-beta 2 glycoprotein 1 antibodies or lupus anticoagulants, protein C, and protein S should be carried out.
- Lumbar puncture always with fluid cytospin analysis and possibly concomitant administration of intrathecal chemotherapy; the initial puncture should be unbloody and thus carried out with great care and when indicated under fluoroscopy performed by an experienced oncologist (bland CSF can be converted to a hemorrhagic CSF, resulting in CNS disease); the puncture is used for assessment of the CNS status; during very high initial leukocyte counts (e.g., >100,000/μL), the lumbar puncture should be postponed a few days until leukocyte counts reach <50,000/μL.
- If applicable, cryoconservation of the sperm or ovaries.

CNS Involvement

A potential CNS involvement can be suspected with a non-traumatic lumbar puncture with a leukocyte count of <5/μL in the CSF fluid but lymphatic blasts in the cytospin. Leukocyte counts ≥5/μL in the CSF and the cytospin after non-traumatic lumbar punctures confirm a CNS involvement.

Testicular Involvement

Testicular involvements clinically manifest as a painless swelling of the testicles. Sonographic examinations and – if necessary – biopsies should be carried out.

Therapy

Initial Patient Admission

Initially a peripheral venous catheter should be established. A comprehensive blood testing is performed including cross blood testing for one to two erythrocyte concentrates depending on the hemoglobin (Hb) value. In order to prevent leukostasis during high initial leukocyte counts, blood products should only be cautiously administered. Hydration with 3 L/m^2 should be applied, whereas an alkalization of the urine is not necessary. Initially a substitution of potassium should be avoided and should be used depending on the measured serum concentrations and the anticipated cell destruction during chemotherapy. Allopurinol should be administered at a dosage of 10 mg/kg body weight per day in two separate doses per os. Rasburicase at a dosage of 0.1–0.2 mg/kg body weight per day as a 30-min infusion should be administered if the patient has very high tumor masses (leukocyte counts >100,000/μL) and/or a mediastinal tumor, as well as high uric acid serum concen-

trations. Leukocyte-depleted erythrocyte concentrates should be only used at Hb values <7–8 g/dL and clinically relevant symptoms of anemia. A maximum Hb of 8 g/dL should be targeted. The transfusion of erythrocytes can induce or worsen headaches. Platelet infusions should be performed at thrombocyte counts <10,000–20,000/μL. A clear indication for the thrombocyte substitution is given with manifest bleeding. A relative indication is given with increasing or pronounced skin bleeding. At thrombocyte counts <10,000/μL, clinically relevant bleeding has to be expected. Fresh frozen plasma (FFP) can be administered at a dosage of 15–20 mL/kg body weight if there is evidence of DIC. An antibiotic prophylaxis has to be started immediately, e.g., with piperacillin/tazobactam 300 mg/kg body weight per day (maximum dose 3×4 g per day). The choice of the specific antibiotic therapy depends on potentially existing infection herds. The planning and preparation for the implantation of a central venous catheter or a port can be started when the patient is in a clinically stable general condition. In pediatric patients with high initial cell counts and/or a large mediastinal tumor, a fast and massive destruction of the tumor cells can occur, and thus a tumor lysis syndrome must be considered.

Chemotherapy

The therapy of the ALL takes 2 years from the first day of therapy initiation. The primary goal of the induction therapy in the course of an intensive therapy is to induce a remission with lymphoblast counts of <5% in the bone marrow and the proof of a normal hematopoiesis. Furthermore, induction therapy aims to rapidly and comprehensively reduce the initial mass of leukemic cells and avoid the persistence or emergence of resistant leukemic subclones as a result of insufficient or ineffective treatment. Induction therapy includes the combination of four different agents: steroids, vincristine, daunorubicin, and L-asparaginase. Consolidation therapy aims to further reduce lymphatic blasts, while re-induction aims to maintain the patient's status. Consolidation therapy includes a combination of different chemotherapeutic agents from those used in the induction therapy. High-dose methotrexate is followed by an obligate folinic acid rescue, reducing remaining leukemic cells in the organs. The intrathecal space is an extra compartment of the body that can hardly be reached by systemic chemotherapy. Therefore, the CNS prophylaxis is administered separately by intrathecal chemotherapy. After 6–9 months of intensive therapy, an individually different amount of lymphatic blasts is in the resting phase of the cell cycle. These cells cannot be destroyed by chemotherapy because most of the cytotoxic agents are only effective against proliferating cells. Therapy is long term taking over 15–18 months to destroy the proliferating cells. Hematopoietic cell transplantation (HCT) is given in about 5% of children <15 years of age who have the primary manifestation of an ALL. It is used in children with a high relapse risk, e.g., in patients with a slow treatment response or with a positive proof of the fusion protein BCR-ABL (Ph+). In these patients – who comprise an aberrant tyrosine kinase activity of the BCR-ABL fusion protein – TKI (e.g., imatinib, dasatinib) are used in the different ALL therapy phases.

Relapse

A relapse of the ALL is observed within the first 5 years after diagnosis in about 10–15% of pediatric patients as a systemic or extramedullary ALL (e.g., CNS, testicles), indicating an intensive chemotherapy. When the treatment response to the initial relapse chemotherapy is only moderate, HSCT is necessary. An HCT is not indicated with late relapses or an isolated relapse (e.g., CNS, testicles). After an intensive re-induction of the intensive therapy, the remission rate is about 90%. An early relapse (occurrence during the therapy phase and during the first 6 months after the end of the therapy) has an unfavorable prognosis with a secondary remission probability of 10–30%, while a late relapse has a remission probability of 40–50%. A CNS relapse is either isolated or combined with a BM or testicular relapse, often accompanied by signs of intracranial pressure such as headache and vomiting and sometimes paresis of the cerebral nerves. In the diagnostic lumbar puncture, a CSF pleocytosis and lymphatic blasts in the cytospin are detectable. If leukemic blasts are not detectable, an MRI of the CNS is necessary to exclude a *meningeosis leucaemica*. Meningeosis leucaemica is characterized by the infiltration of the meninges and the subarachnoid space by malignant cells (leukemic blasts) as a consequence of leukemia. The therapy is carried out intrathecally in addition to a systemic induction chemotherapy and cranial radiotherapy until a CNS remission is achieved. The side effects of the radiotherapy include intellectual impairments (concentration disorders), growth disorders, and neurotoxicity. Intrathecal chemotherapy is administered via an Ommaya reservoir, enabling a uniform mixing of the cerebrospinal fluid and the administered cytostatics. An isolated testicular relapse is often the precursor of a systemic relapse and normally occurs as a painless, rough swelling of one or both testicles. A biopsy of the testicles and intensive, systemic chemotherapy are then usually indicated. *Ovarian* leukemic *relapse* in female patients is much rarer than testicular relapses in male patients. Other local relapses are lymph nodes, skin, eyes, or other extramedullary organs (thymus, mastoid).

Diagnosis of Minimal Residual Disease (MRD)

The diagnosis of the MRD is performed by a molecular monitoring of the residual tumor burden enabling individual treatment of ALL. Through analyzing appropriate desoxyribonucleic

acid (DNA) markers, one leukemic cell may usually be detected in 10,000 (10^{-4}) up to 100,000 (10^{-5}) leukocytes. Thus, a distinctly more sensitive detection of leukemic cells is possible when compared to conventional methods such as the cell determination of cell morphology, cytogenetics, or immune phenotyping. As a marker system, clone-specific immunoglobulin (Ig) or T cell receptor (TCR) gene rearrangements are used. Ideally, two different gene probes with a detection limit of 10^{-4} are used in each patient. This method combines three diagnostic tools: a molecular characterization of the leukemia at the time of diagnosis to identify molecular clonal IgR/TCR gene rearrangements (identification of a DNA fingerprint of the leukemia), establishing patient-specific quantitative polymerase chain reaction (PCR) assays including sensitivity and specificity testing, as well as quantifying the residual tumor burden in the course of the disease. During the intensive ALL therapy phase, MRD diagnosis is performed at different time points depending on the protocol used, and it determines individually the intensification or reduction of the according therapy.

Long-Term Effects and Follow-Up Care

The follow-up (FU) aims at the early detection and treatment of an ALL relapse and possible long-term effects, as well as helping the patient and his/her family to cope with physical, psychological, or social problems. Special rehabilitation measures help to support and accelerate the recovery and healing process. It is thus highly recommended to perform the proposed FU examinations, providing the security not only of detecting a relapse of the disease but also related adverse events and secondary diseases. The major objective of the FU is the physical examination, blood testing, and medical consultation. FU care is performed once a month in the 1st year after the end of the therapy, every 3 months in the 2nd and 3rd year, and then every 6 months thereafter. Furthermore, different laboratory checks are conducted after the end of the therapy to analyze the organ function (liver, kidney, thyroid), detect virus infections, and analyze the hormone status (growth hormones, sex hormones). Every 2–3 years, the cardiac function is analyzed by echocardiography. The FU examinations are mandatory in the first 5 years after the end of the therapy, because the relapse risk is highest during this period of time, especially during the first 2 years. Moreover, an annual FU check is also necessary after 5 years since about 10% of the relapses occur after this time. Likewise, related late effects may only occur after this period of time.

Acute Myeloid Leukemia (AML)

Definition

The term AML includes leukemia of a heterogenic group of hematologic progenitor cells of the myeloid, monocytic, erythrocytic, and megakaryocytic cell lines. It leads to a partially massive expansion of immature progenitors of myelopoiesis in the bone marrow and in the majority of cases also in the peripheral blood, detectable as a leukocytosis with repression of the normal hematopoiesis. AML is a heterogeneous disease considering its pathogenesis, immune phenotype, genetic mutations, or genome patterns, as well as its treatment response. AML is defined as either a de novo AML or a secondary AML. Both forms act separately concerning their biological features and the treatment response. A secondary AML develops from a previous hematologic illness – e.g., MDS – and normally responds worse to the treatment.

Incidence

The incidence of AML is between 15% and 20% of all pediatric leukemia. There is a regular distribution by age with a slightly increased incidence in children under 2 years of age and during adolescence. There is no sex preference. A genetic disposition is given in patients with trisomy 21 or Fanconi's anemia [44].

Prognosis

The prognosis of pediatric and adolescent patients with AML has significantly improved due to the immense advancements of therapy regimens in the last 30 years. The currently used modern diagnostic methods and intensive, standardized combination chemotherapy regimen enable a 5-year survival of over 75% in pediatric and adolescent patients with AML [1, 45].

Thirty percent of all children with AML currently cannot be healed. Most of these children experience an AML relapse. The individual prognosis for each of these patients mostly depends on the AML subtype and the treatment response. In ~10% of pediatric patients, no or only a poor treatment response is observed, and an initial remission cannot be achieved. Favorable prognostic factors are M1 with Auer bodies, promyelocytic leukemia (M3), M4 with eosinophilia and favorable cytogenetics (t(15;17), t(9;11), inv(16), t(8;21)) or normal karyotype, and fast treatment response (MRD at the end of the induction therapy and remission for >1 year after the end of therapy). Unfavorable prognostic factors are high leukocyte counts >100,000/μL; CNS involvement; FAB (French-American-British classification system) M0, M6, and M7; unfavorable cytogenetics such as trisomy 8, aberration of chromosome 5 or 7, t(6;9), and t(9;22); babies with 11q23; complex cytogenetic aberrations such as monosomy 5, 7(5q), and 3q; MRD positivity; previous hematologic diseases (e.g., MDS); secondary AML; or duration of remission <1 year.

Pathogenesis

The AML derives from a pathological proliferation of clonal myeloid cells that usually belong to the highly proliferative progenitor pool (CD34+/CD38+) or rare to the stem cell pool (CD34+/CD38-). There are AML-specific genetic aberrations like the translocations t(8;21) and t(15;17) or the inversion inv(16). Numeric aberrations such as trisomy 8 and monosomy 7 or complex aberrations with more than three recurrent chromosomal aberrations with a single clone are also important. All of these aberrations comprise an important prognostic role. The next-generation sequencing (NGS) analysis of 200 AML patients could detect an average of five recurrent aberrations per patient. The most frequent mutations were found in the genes FLT3, NPM1, DNMT3A, and IDH1/2 in at least 20% of the patients. Almost all patients showed at least one mutation in the functional group of nine functional gene groups that are critical for the malignant transformation: (1) activating mutations of the signal transduction (e.g., FLT3, KIT, KRAS, NRAS), (2) mutations of myeloid transcription factors (e.g., RUNX1, CEBPA), (3) fusions of transcription factor genes (e.g., PML-RARα, MYH11-CBFB), (4) mutations of chromatin modifiers (e.g., MLL-PTD, ASXL1), (5) mutations of the cohesin complex (e.g., SMC1S), (6) mutations of the spliceosome, (7) mutations of tumor suppressor genes (e.g., TP53, WT1), (8) NPM1 mutations, and (9) mutations of the DNA methylation genes (e.g., TET1, TET2, IDH1, IDH2, DNMT3B, DNMt1, DNMT3A) [46–48].

Diagnosis

The AML is diagnosed by blood tests such as complete blood count, liver and kidney function panels, cell decomposition parameters (e.g., LDH), urea, potassium, phosphate, and blood chemistry. These parameters provide important information about the specific cell counts and how well the organs are functioning. The blood cells are viewed under a microscope to check for abnormal shapes or sizes. A marrow aspiration is used to detect and determine relative numbers of myeloid blasts in the bone marrow. A lumbar puncture is used to analyze the CSF to identify an affection of the CNS with myeloid blasts.

Differential Diagnosis

For differential diagnosis, virus infections (e.g., parvovirus B19, Epstein-Barr virus (EBV), cytomegalovirus (CMV), human immunodeficiency virus (HIV)), vitamin B12 or folic acid deficiency, anemia, juvenile rheumatoid arthritis, acquired neutropenia, autoimmune cytopenia, megaloblastic anemia, aplastic anemia, ALL, AML of unknown cell line specificity, leukemic lymphoma, MDS, myeloproliferative syndromes (MPS), JMML, or CLM have to be excluded.

Typing

Morphology and Cytochemistry

Diagnosis of morphology and cytochemistry is based on microscopic analyses of the bone marrow smear. Characteristics such as the detection of Auer rods enable classifying blasts of the myeloid cell lines. Auer bodies are fine, rod-shaped granules or large, oval to elliptical inclusions (Auer bodies) in the cytoplasm of immature leukemia cells. In the majority of cases, the distinction between an AML and ALL is possible by the French-American-British (FAB) classification system, if necessary with an additional cytochemical analysis (peroxidase and esterase staining, PAS reaction) (Table 35.6 and Figs. 35.5, 35.6, 35.7, 35.8, 35.9, 35.10, 35.11, 35.12, 35.13, 35.14, 35.15, 35.16, and 35.17).

The current WHO classifications – which have included more balanced translocations and inversions as individual entities (e.g., t(15;17), t(8;21), inv(16), t(9;11), inv(3)/t(3;3), t(6;9), t(1;22)), as well as two molecular genetically defined

Table 35.6 Morphology during different FAB stages

FAB stage	Morphology
M0	Myeloblasts without granules
	No Auer rods
	<3% of the blasts are peroxidase positive
M1	Myeloblasts with or without granules; little differentiated
	Blasts with a thin cytoplasmic seam and fine chromatin with distinct nucleoli
	Auer rods possible
	≥3% of the blasts in the bone marrow are peroxidase positive
M2	Large myeloblasts with partially abundant cytoplasm and azurophil granule
	Individual myelocytes
	Auer rods
M3	Promyelocytes
	Blasts are rich of azurophil granules
	Distinctly granulated
	Auer rod clusters
M4	Myeloblasts and promyelocytes >20%
	Auer rods possible
	Unspecific esterase staining: differentiation from other AML subtypes
M5a	Large monocytic blasts
	>80% of blasts in the bone marrow are monocytic blasts
	No Auer rods
M5b	Monocytic blasts, promonocytes, and monocytes
	>80% of blasts in the bone marrow are monocytic blasts
	Monocytosis in the peripheral blood
	No Auer rods
M6	Megaloblastic erythropoiesis >50%
	Myeloblasts >30%
	Auer rods
M7	Megakaryoblasts
	No Auer rods

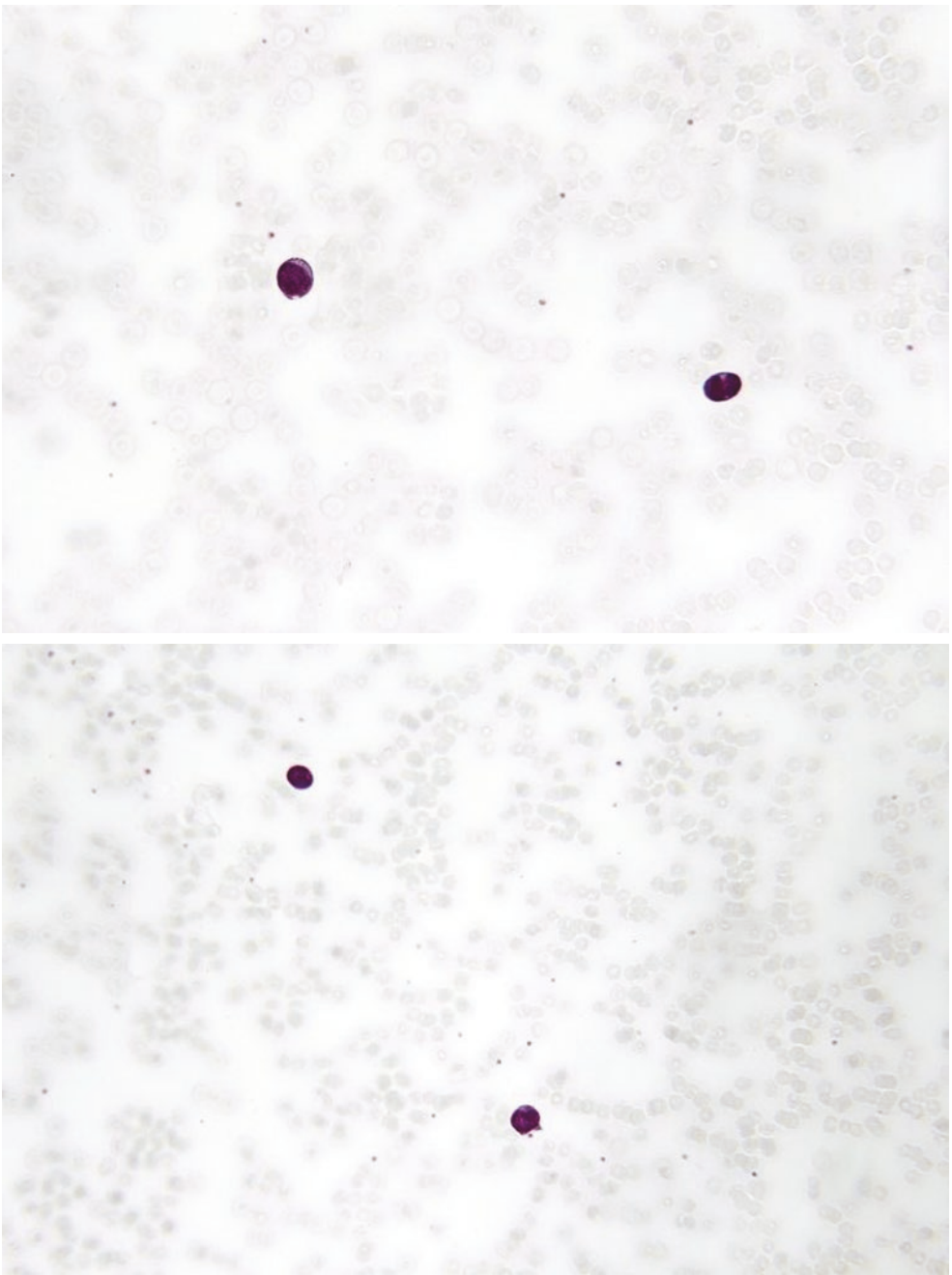

Fig. 35.5 Acute myeloid leukemia M0 (AML-M0). Undifferentiated myeloid leukemia: <3% of the blasts are peroxidase positive

entities (AML with NPM1 mutation and AML with bilateral CEBPA mutation) – resemble the improved understanding of the molecular pathogenesis of the AML (Table 35.7). Furthermore, an AML with a myelodysplasia-associated cytogenetic alteration that includes a multitude of unbalanced and balanced aberration has been defined as another subgroup of the AML.

Immune Phenotyping

The immune phenotyping in the diagnosis of AML is rather of confirmative nature, but can provide important additional information in case of doubt. The expression of membrane-bound surface markers on leukemia cells can be analyzed using fluorescence-stained monoclonal antibodies. In most cases, differentiation antigens are analyzed that are also expressed in the course of the normal hematopoiesis and summarized in the CD nomenclature (Table 35.8). The biphenotypic leukemia expresses myeloid and lymphatic markers. A necessary predisposition is the expression of at least one surface marker of the other line (myeloid or lymphatic). Mixed leukemia forms that contain blasts with more than one phenotype have to be distinguished. Furthermore,

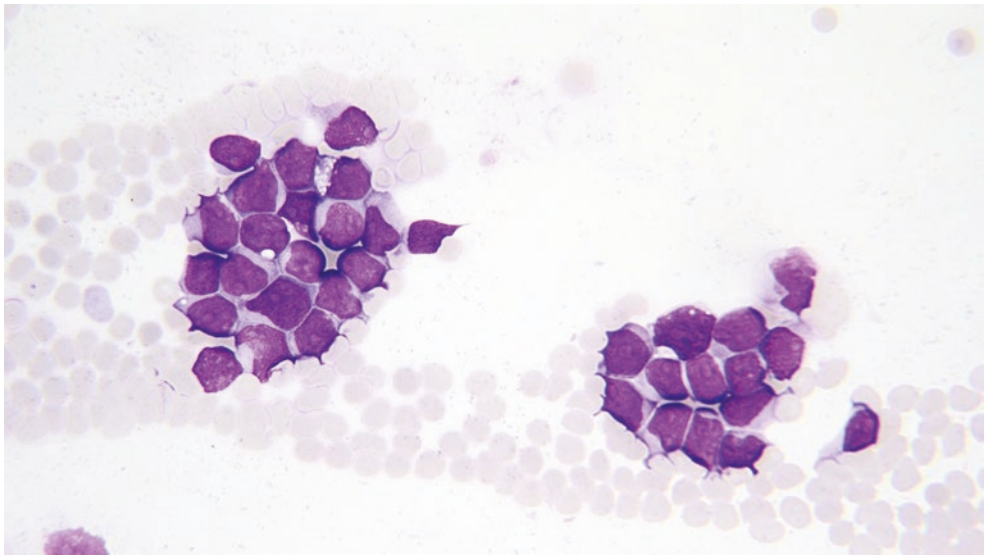

Fig. 35.6 Acute myeloid leukemia M1 (AML-M1) without Auer bodies. Myeloblastic leukemia without maturation: the blasts are little differentiated and have a rather slim cytoplasmic seam, fine chromatin, and distinct nucleoli; in the bone marrow, at least 3% of the blasts have to be peroxidase positive

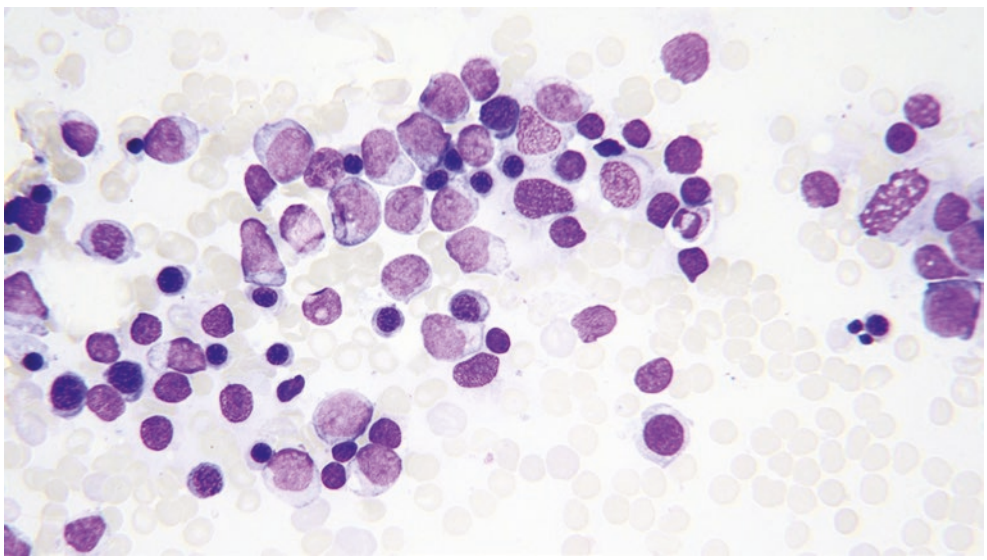

Fig. 35.7 Acute myeloid leukemia M1 (AML-M1) with Auer bodies. Myeloblastic leukemia with differentiation: the blasts are large with partially abundant cytoplasm with azurophil granule; Auer rods are common; ca. 20% of the AML M2 comprise a translocation (8;21)

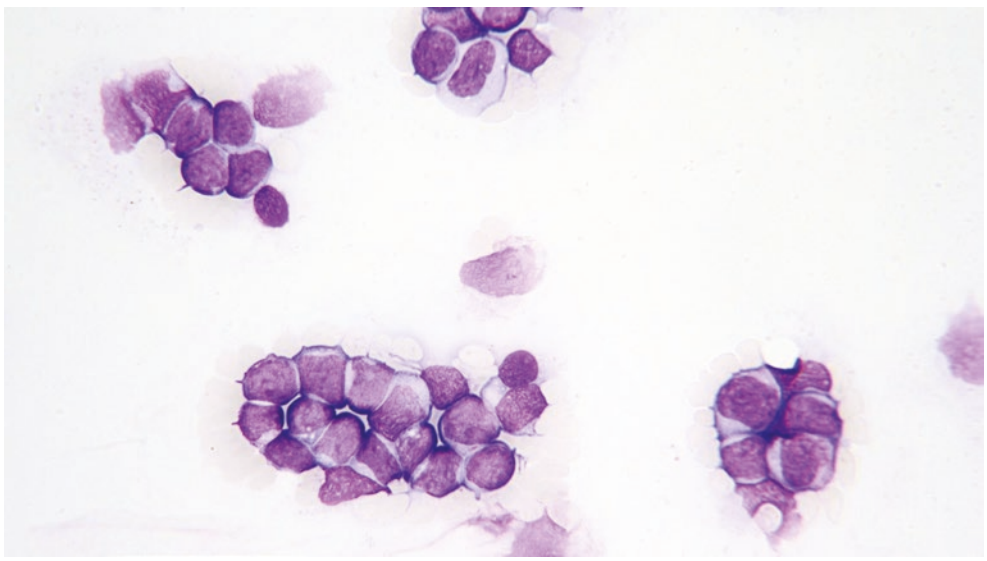

Fig. 35.8 Acute myeloid leukemia M2 (AML-M2) without Auer bodies. Myeloblastic leukemia with differentiation: the blasts are large with partially abundant cytoplasm with azurophil granule; Auer rods are common; ca. 20% of the AML M2 comprise a translocation (8;21)

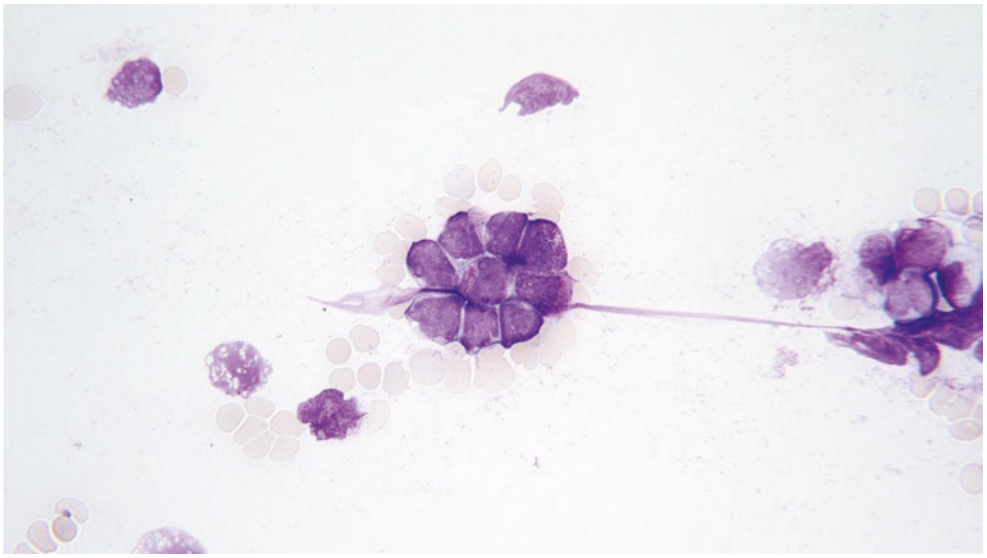

Fig. 35.9 Acute myeloid leukemia M3 (AML-M3) with Auer rod clusters. Promyelocytic leukemia: the blasts are rich of azurophil granule; often numerous Auer rods; morphologically, the typical hypergranulated form can be differentiated from the rarer microgranulated variant M3v (fine granule and a bilobular nucleus); the enzymes of the granules released during cell death can lead to the feared disseminated intravascular coagulation; this complication has a high mortality but has become considerably rarer due to the use of all-trans retinoic acid (ATRA). ATRA in combination with chemotherapy has distinctly improved survival rates in patients with M3. AML M3 comprise a characteristic translocation (15;7)

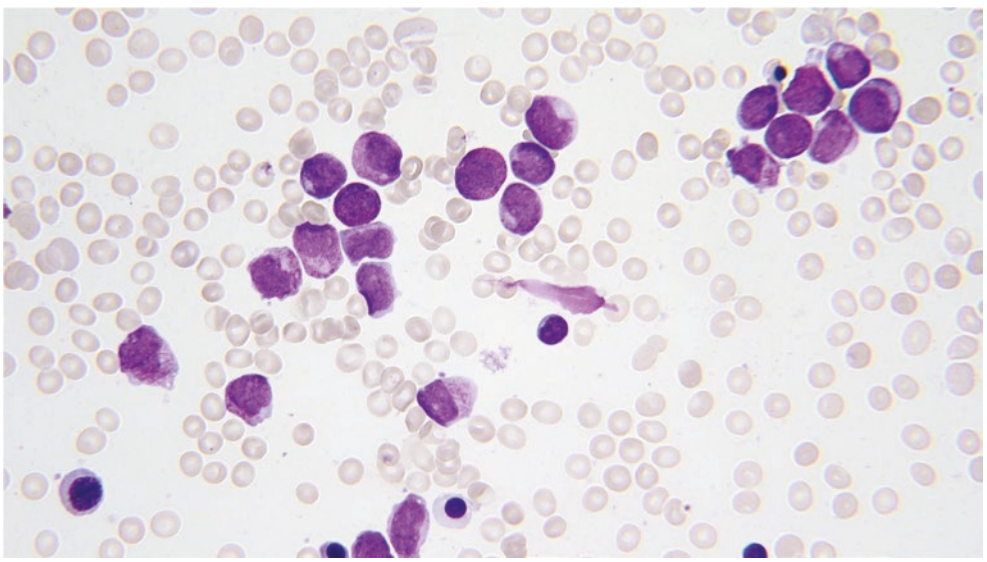

Fig. 35.10 Acute myeloid leukemia M3 (AML-M3) with Auer rod clusters. Promyelocytic leukemia: the blasts are rich of azurophil granule; often numerous Auer rods; morphologically, the typical hypergranulated form can be differentiated from the rarer microgranulated variant M3v (fine granules and a bilobular nucleus); the enzymes of the granules released during cell death can lead to the feared disseminated intravascular coagulation; this complication has a high mortality but has become considerably rarer due to the use of all-trans retinoic acid (ATRA). ATRA in combination with chemotherapy has distinctly improved survival rates in patients with M3. AML M3 comprise a characteristic translocation (15;7)

there are leukemia subtypes that express both M6 and M7 markers and are thus referred to as erythro-megakaryocytic leukemia.

Cytogenetic Typing

In most AML patients, numeric and structural chromosomal aberrations can be detected in the leukemia cells. Some of these aberrations are closely related to specific morphologic and clinical subtypes or, respectively, define these subtypes. The most common numeric aberration in the AML is the trisomy 8, while the most common structural aberration is t(8;21). The aberration t(15;17) is highly specific for the promyelocytic leukemia, whereas the inversion inv(16) is characteristic for M4E0. Moreover, the aberration t(9;22) (the

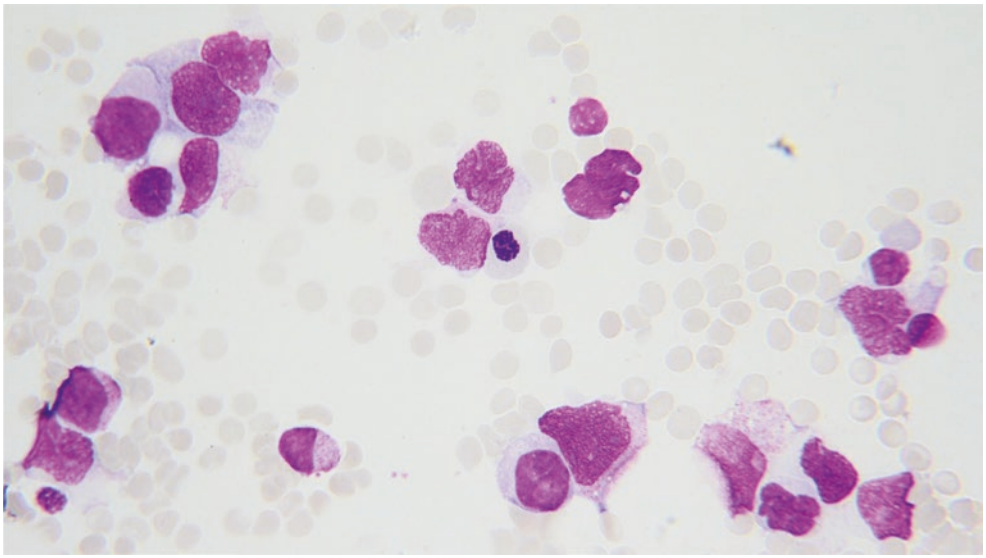

Fig. 35.11 Acute myeloid leukemia M4 (AML-M4). Myelomonocytic leukemia: AML with myeloid and monocytic maturation; in 10% of the patients, a gingiva hyperplasia occurs; for differentiation of other forms of the AML, the unspecific esterase staining is used

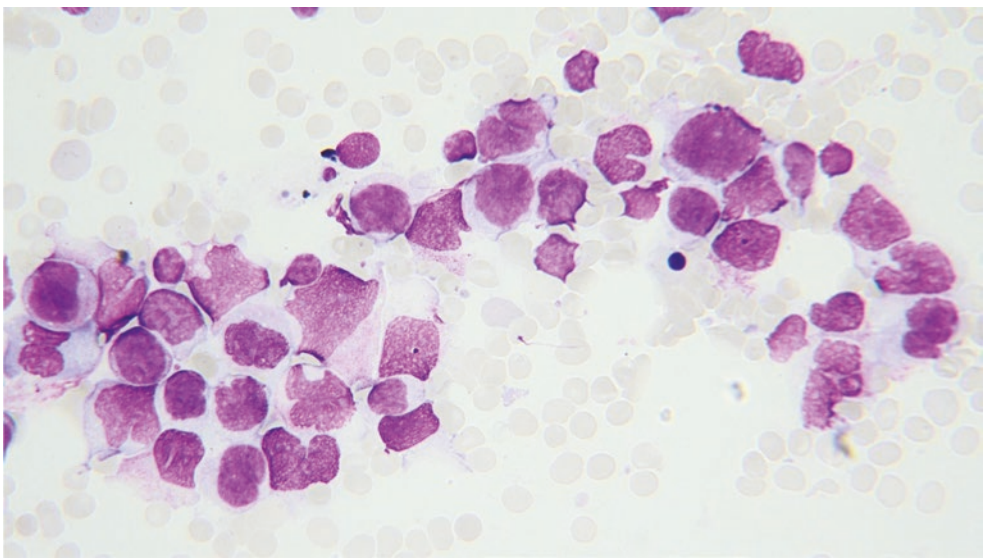

Fig. 35.12 Acute myeloid leukemia M4 (AML-M4). Myelomonocytic leukemia: AML with myeloid and monocytic maturation; in 10% of the patients, a gingiva hyperplasia occurs; for differentiation of other forms of the AML, the unspecific esterase staining is used

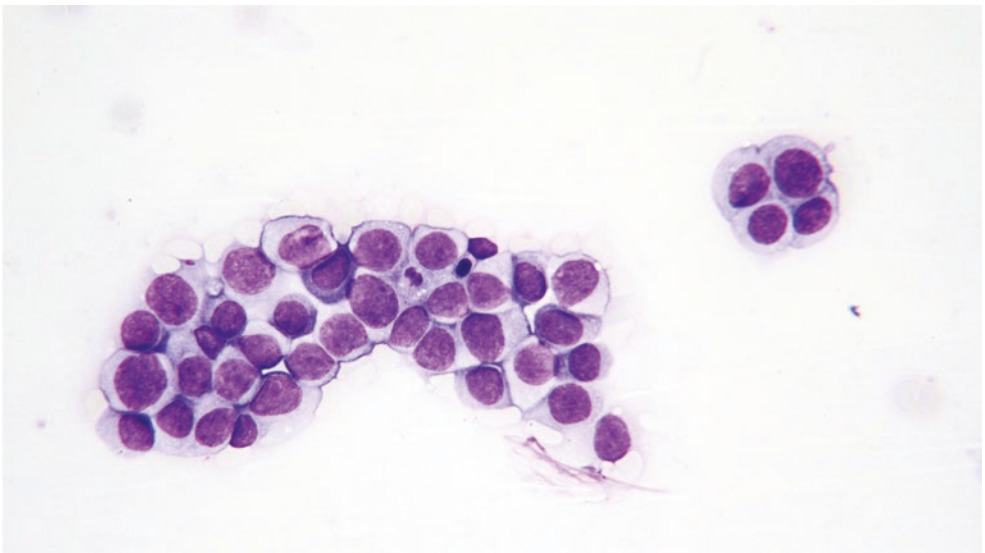

Fig. 35.13 Acute myeloid leukemia M5 (AML-M5). Monocytic leukemia: infiltrations of the skin, mucous membranes, lungs, colon, meninges, lymph nodes, bladder, and larynx occur often. Over 80% of the blasts in the bone marrow are monocytic. Monocytic leukemia is differentiated into forms without (M5a) or with (M5b) maturation

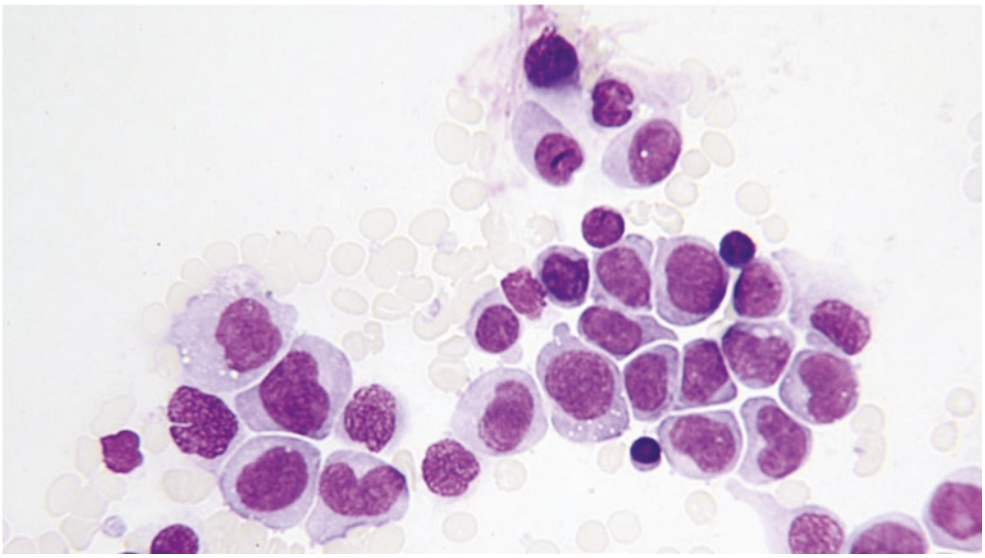

Fig. 35.14 Acute myeloid leukemia M5 (AML-M5). Monocytic leukemia: infiltrations of the skin, mucous membranes, lungs, colon, meninges, lymph nodes, bladder, and larynx occur often. Over 80% of the blasts in the bone marrow are monocytic. Monocytic leukemia is differentiated into forms without (M5a) or with (M5b) maturation

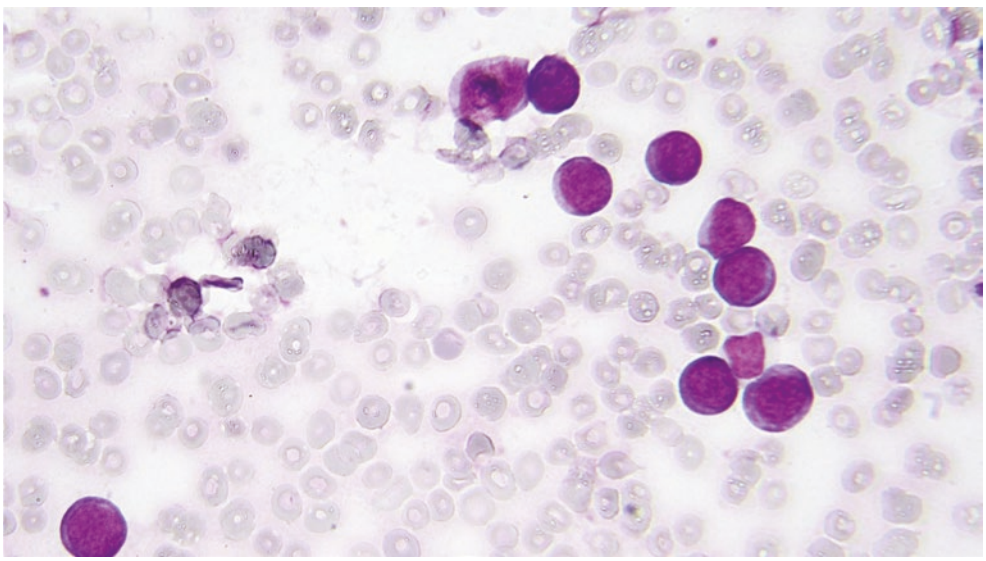

Fig. 35.15 Acute myeloid leukemia M6 (AML-M6). Acute erythroid leukemia: rare; over 50% of the nucleated cells in the bone marrow have to belong to the red cell line and over 30% of the other cells to blasts of the type M1 or M2

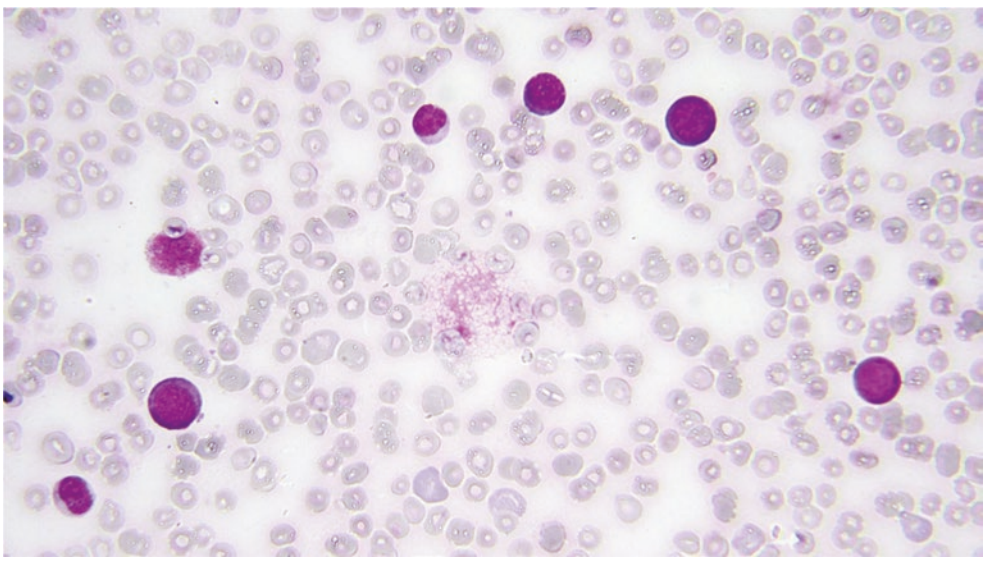

Fig. 35.16 Acute myeloid leukemia M6 (AML-M6). Acute erythroid leukemia: rare; over 50% of the nucleated cells in the bone marrow have to belong to the red cell line and over 30% of the other cells to blasts of the type M1 or M2

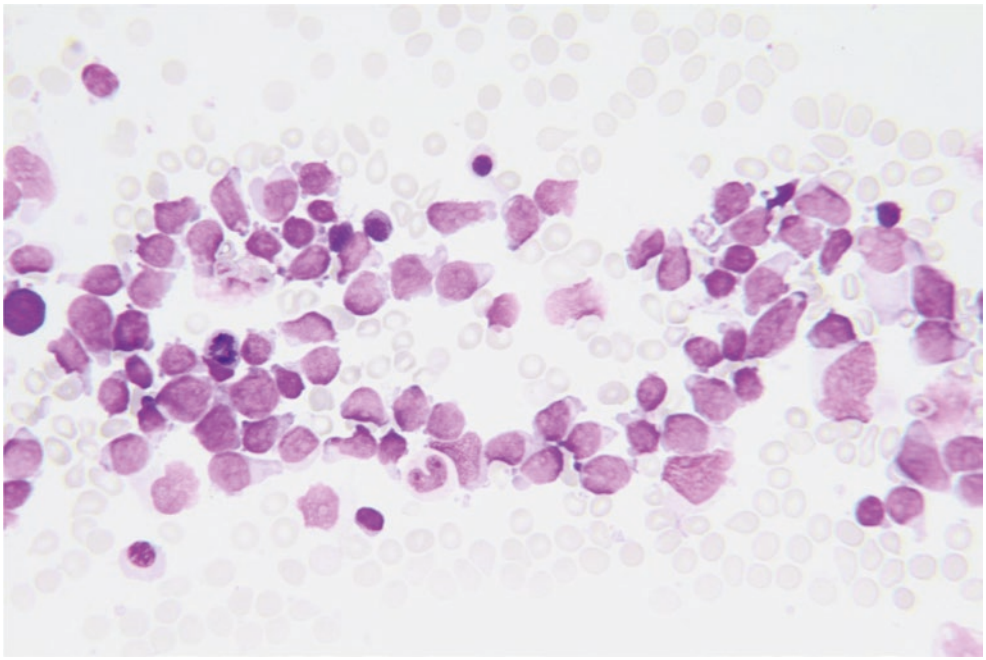

Fig. 35.17 Acute myeloid leukemia M7 (AML-M7). Megakaryoblastic leukemia: rare; today, the classification of leukemia is increasingly based on immune phenotypic, cytogenetic, and molecular biological analyses (e.g., proof of translocations by RT-PCR)

Table 35.7 WHO nomenclature of the myeloblast classification

Disease	Description
AML with genetic mutations and anomalies	AML with t(8;21) (q22;q22), RUNX1-RUNX1T1 (CBFA/ETO)
	AML with inv(16) (p13;q22) or t(16;16) (p13;q22); CBFB-MYH11
	APL with t(15;17) (q22;q11-12); PML-RARα
	AML with t(9;11) (p22;q23); MLLT3-MLL
	AML with t(6;9) (p23;q34); DEK-NUP214
	AML with inv(3) (q21;q26.2) or t(3;3) (q21;q26.2); RPN1-EVI1
	AML (megakaryoblastic) with t(1;22) (p13;q13); RMB15-MKL1
	AML with NPM1 mutation
	AML with CEBPA mutation
	AML with characteristics of myelodysplasia
	Therapy-related myeloid neoplasms
Not further specifiable AML	AML with minimal differentiation
	AML without maturation signs
	AML with maturation signs
	Acute myelomonocytic leukemia
	Acute monoblastic and monocytic leukemia
	Acute erythroblastic leukemia
	Acute megakaryoblastic leukemia
	Acute basophile leukemia
	Acute panmyelosis with myelofibrosis
	Myeloid sarcoma
Myeloid proliferation during Down syndrome	Transient abnormal myelopoiesis
	Myeloid leukemia during Down syndrome
	Blastic plasmacytoid dendritic cell neoplasm

AML acute myeloid leukemia, *APL* acute promyelocytic leukemia

Table 35.8 Immune phenotyping of acute myeloid leukemia subtypes

	M0	M1/M2	M3	M4Eo	M5	M5	
		t(8,21)	t(15,17)	inv(16)		t(9,11)	M7
MPO	+/–	+	–	+	–/+	–	–
CD2	–			+/–			
CD13	+/–	+	+	+	+/–	–	+/–
CD14	–	–	–	+/–	+/–	–	–
CD15	–	+/–	–/+	+/–		+	–
CD19	–	+/–					
CD33	+/–	+/–	+	+	+	+	+/–
CD34	+/–	+/–	–	–/+			
CD56		+/–					
CD61	–	–	–	–	–	–	+
CD64	–	–	+/–	+	+	+	
CDw65	–/+	+/–	–/+	+	+/–	+	+/–
CD117	+/–	+/–	–/+	+/–	–/+		
HLA-DR	+/–	+	–	+	+	+	+/–

CD cluster of differentiation, *HLA-DR* human leukocyte antigen DR, *MOP* myeloperoxidase

Philadelphia chromosome) can be detected in AML (Table 35.9).

The FAB classification system has been complemented by the WHO system, which additionally includes immune phenotypic, cytogenetic, and molecular characteristics (Table 35.7).

Clinical Symptoms

The symptoms of AML are unspecific in pediatric and adolescent patients and are mostly ascribed to the repression of

Table 35.9 Cytogenetic anomalies in children with AML

FAB stage	Incidence (%)	Chromosomal aberration	Affected gene
M1/M2	20–30	t(8;21)	ETO-AML1
M3	15–18	t(15;17)	PML-RARα
	<1	t(11;17)	RARα
M4 or M5	14–32	t(9;11)	AF9-MLL
M5	<1	t(11q23)	MLL
M5	<1	t(1b;11)	AF10-MLL
M5	<1	t(11;17)	A17-MLL
M6	<6		
M7	3	t(1;22)	

FAB French-American-British nomenclature

the normal hematopoiesis in the bone marrow or directly by high concentrations of blasts. Thus, most patients are recognized due to anemia-based pale skin, increased hematomas and petechiae caused by the thrombocytopenia, or infection due to the neutropenia and lymphopenia. High blast counts lead to problems with the blood viscosity, commonly starting with pulmonary symptoms, or blood coagulation disorders increasing the risk of severe bleeding. Especially in cases of monoblastic leukemia, multiple skin infiltrations can occur. A hyperplasia of the gingiva should give reason for further hemato-oncologic diagnosis. Especially AML subtypes with the translocation t(8;21) are associated with other extramedullary manifestations such as orbital tumors or myelosarcoma and chloroma, which can be localized everywhere else.

Hyperleukocytosis and Leukostasis

High leukocyte counts >200,000/μL can lead to a leukostasis, causing intravascular clotting. Small vessels clog inducing brain infarction or bleeding, especially in the lungs and the CNS, as well as pulmonary insufficiency with tachypnea, dyspnea, or hypoxia. Symptoms of a CNS involvement are stupor, aphasia, ataxia, or nystagmus. Furthermore, deterioration of visual acuity, double vision, and papilledema can occur.

In M5-AML, the leukostasis already occurs at leukocyte counts of >100,000/μL owing to the size of the monocytes. The diagnosis is made by blood testing (electrolytes, kidney parameters, uric acid, blood coagulation parameters, and blood gas analysis), morphology, and chest X-ray. The therapy includes a fast cytoreduction by leukapheresis or exchange transfusion. Hydration and administration of rasburicase are used as tumor lysis syndrome prophylaxis [15].

Bleeding

Besides thrombocytopenic bleeding, a coagulopathy often occurs, causing bleeding of the mucosa (e.g., epistaxis, bleeding of the gingiva), the gastrointestinal tract, or the CNS. The coagulopathy is accompanied by a disseminated intravascular activation of the blood coagulation (DIC), also caused by infection and/or increased coagulation activating cytoplasmic proteins of the myeloid cells (e.g., thromboplastin), which is especially seen in promyelocytic leukemia (M3). The therapy includes a substitution of the thrombocytes at thrombocyte counts <20,000/μL. During severe anemia and Hb <6 g/dL, Hb values are corrected with erythrocyte transfusions.

Infections

The number of functioning neutrophils is regularly $<1 \times 10^9$/L, and fever and bacteremia frequently occur. The risk of fungal infections is very high owing to the long-lasting period of neutropenia/aplasia during the initial therapy.

Therapy

At initial leukocyte counts >50,000/μL or substantial organ hypertrophies, a prior therapy with cytarabine (40 mg/m^2 per day; IV) and possibly with 6-thioguanine (40 mg/(m^2 per day); by mouth) should be administered. If a blast reduction cannot be achieved within 3 days, induction chemotherapy should be started immediately. If the bleeding risk persists, induction therapy should be modified, e.g., with half of the L-DNR dose. Overall, the duration of the prior therapy should not exceed 7 days.

CAVE:

- Anthracyclines should not be used immediately.
- A diagnostic lumbar puncture should be performed only after blast reduction.
- Antifungal prophylaxis should be started when the patient is stabilized (e.g., with voriconazole or posaconazole orally between the chemotherapy courses).

The basis for the AML therapy is intensive chemotherapy. The aim of induction therapy is a complete remission with an eradication of the pathologic cell population to <5% blasts in the bone marrow, a normalization of the blood counts, and an elimination of all symptoms of the disease.

Chemotherapeutic treatment includes therapy courses for several days, which are repeated multiple times (two to three times). Important agents are cytarabine and the anthracyclines daunorubicin or idarubicin. In some cases, thioguanine is additionally given. When a complete remission is achieved (often after one to two therapy courses), post-remission therapy is started, including consolidation therapy (high-dose cytarabine) and maintenance therapy (all-trans retinol, 6-mercaptopurine, and methotrexate) in case of an

acute promyelocytic leukemia. An allogeneic HCT can be considered as an alternative treatment option.

An intrathecal cytarabine administration is used as CNS prophylaxis in combination with methotrexate and prednisolone. Some protocols contain a maintenance therapy for 1 year after intensive therapy. The AML treatment with high-dose chemotherapeutics aims to eliminate the malignant clone, enabling a regeneration of the normal hematopoiesis. Therapy optimization trials could achieve a long-term event-free survival of 40–75%.

Allogeneic Hematopoietic Cell Transplantation

Several studies have shown that the allogeneic HCT has significantly improved survival rates in AML. However, in AML patients with favorable prognosis and in first remission, the allogeneic HCT does not seem to have a significant advantage, as opposed to patients with moderate risk AML. In patients with a high-risk AML, the HCT is used due to the poor prognosis and despite an unclear benefit. A possible antileukemic effect by the donor immune system – the graft-versus-leukemia effect (GvL) – can lead to prognostically improved long-term results in some AML subtypes and in combination with a supportive therapy and a graft-versus-host disease (GvHD) prophylaxis. Long-term effects after transplantation include the chronic graft failure, bone marrow suppression, pulmonary insufficiency, growth retardation, sterility, and the risk of secondary malignancies. The long-term leukemia-free survival ranges between 50% and 80%, depending on the individual prognostic factors of the AML. In cases without an available HLA-identical family donor (matched family donor, MFD), an identical or partially identical foreign donor (matched unrelated donor, MUD; mismatched unrelated donor MMUD), an umbilical cord blood or a haploidentical family donor can be considered for HCT.

Relapse

About 30% of pediatric and adolescent patients with AML experience a relapse of the disease [49]. The chances of complete recovery after an AML relapse are generally more unfavorable than in patients with an initial manifestation. The 5-year survival rate is currently about 40% [50]. However, the prognosis of an AML relapse strongly depends on the specific time of its occurrence after initial manifestation and the treatment response. Pediatric patients with a late relapse (>1 year after end of therapy) usually have a better prognosis than patients with an early relapse. In most but not all cases, a late relapse has a better treatment response than an early relapse. A second remission can be induced chemotherapeutically in more than half of pediatric patients with an AML relapse. However, for the maintenance of the remission, an allogeneic HCT is usually necessary. The AML relapse can be diagnosed by MRD analysis when MRD markers could be detected at the time of the initial diagnosis.

Long-Term Side Effects and Follow-Up (FU)

The FU aims at the early detection and treatment of an AML relapse and possible long-term effects, as well as helping the patients and their families to cope with physical, psychological, or social problems. Special rehabilitation measures help to support and accelerate the recovery and healing process. The treatment of AML in pediatric and adolescent patients requires intensive treatment regimens. Not only high-dose medication and radioactive irradiation but also the disease itself and its related stresses can induce physical and psychological side effects that occur only after the end of the therapy as long-term effects. Severe side effects in these patients are secondary malignancies, cardiotoxicity, and as a result of the stem cell transplantation a chronic GvHD. The cumulative incidence rate for secondary malignancy after 20 years is approximately 2%. However, about half of long-term survivors report chronic health problems after the treatment. Severe, life-threatening diseases are three times more likely in these patients compared with the normal control population. A late cardiotoxicity is expected in about 5% of the patients. However, its clinical manifestation is only seen in half of the affected patients. Statements on the fertility can only be made with difficulty: 14% of female patients treated with chemotherapy only showed a significant reduction of the anti-Müllerian hormone as a sign of an impairment of fertility. It is highly recommended to perform the proposed FU examinations, providing the security not only of detecting a relapse of the disease but also related adverse events and secondary diseases. Therefore, regular clinical examinations as well as blood and bone marrow screenings are necessary. Clinical suspicions of a relapse or suspicious blood screening results indicate a bone marrow puncture. Since the majority of relapses occur within 18–24 months after achieving remission, blood screenings should be performed every 1–3 months within the first 2 years and every 3–6 months within 3–5 years after the end of therapy.

Myelodysplastic Syndromes (MDS)

Definition

MDS do not present a particular pathology but rather summarize conditions that are characterized by a clonal malignancy of the multipotent stem cell. MDS present with cytopenia of at least one hematopoietic cell line and dysplastic transformations of at least two hematopoietic cell lines. A secondary MDS may occur after chemo- or radiotherapy.

Incidence

The incidence of primary MDS is estimated to about 4% of all pediatric malignant blood diseases. The median age at diagnosis of MDS is 9 years. Secondary MDS are observed in about 7–18% of pediatric MDS patients.

Prognosis

Pediatric patients with MDS who received a reduced intensity conditioning regimen have a 3-year survival of about 84%.

Pathogenesis

About 90% of cases are primary MDS. The pathogenic mechanism of primary MDS is mostly unclear. However, it is assumed that MDS derive from acquired, multipotent hematopoietic progenitor cells that comprise several genetic alterations. These alterations are seen in 40–50% of MDS patients with genetic modifications in the bone marrow cells. Secondary forms are caused by defects of the hematopoietic blasts that occur in the course of a radio-oncological treatment, chemotherapy, or exposure to other physical or chemical stressors (e.g., organic solvents). Some of the patients comprise a higher risk of developing AML.

Diagnosis

The diagnosis of MDS is made by blood testing. Usually, pancytopenia is present and reticulocyte counts are reduced. A bone marrow aspiration is used for detecting blasts and signs of dysplasia; cytogenetic and mutational analyses and a bone marrow biopsy enable immunohistochemical phenotyping to complete the diagnosis. Involvement in the CNS is analyzed by lumbar puncture and detection of blasts in the cerebrospinal fluid.

Differential Diagnosis

At the beginning of the disease, other underlying causes for the cytopenias should be excluded, and the plasma levels of folic acid and vitamin B12 and the iron status including ferritin should be determined. In order to exclude a vitamin B deficiency, homocysteine levels in the serum and urine (increased during vitamin B12 deficiency) as well as methylmalonic acid levels in the serum or urine (increased during vitamin B12 or folic acid deficiency) should be analyzed. Viruses of the herpes group (e.g., CMV, EBV, or human herpes virus 6 (HHV6)) but also parvovirus B19 and HIV6 can present MDS-like clinical pictures. Signs of an intravascular hemolysis such as the increase of LDH and bilirubin as well as reduced haptoglobin hint at a hemolytic anemia, whose cause needs to be further clarified. In MDS these findings arise due to intra-bone marrow RBC destruction. Furthermore, increases of fetal Hb (HbF) should be considered as an indicator for MDS.

Classification

The blast count in the bone marrow is used as a criterion for classifying different MDS forms and differentiating from AML. The occurrence of blasts marks a progression of the following subtypes: refractory anemia (also, refractory cytopenia, RC) with less than 5% blasts in the bone marrow and refractory anemia with excess of blasts (EB, 5–20% blasts). Blast counts of over 20% in the bone marrow indicate a transition to AML (Table 35.10).

Table 35.10 Characteristics of myelodysplastic syndromes subtypes

Classes	Number of dysplastic cell lines	Number of cytopenia cell lines	Percentages of blasts of bone marrow (BM) and peripheral blood (PB)
MDS with single lineage dysplasia (MDS-SLD)	1	1 or 2	BM <5%, PB <1%, *without Auer rods*
MDS with multilineage dysplasia (MDS-MLD)	2 or 3	1–3	BM <5%, PB <1%, *without Auer rods*
MDS with ring sideroblasts (MDS-RS)			
MDS-RS with single lineage dysplasia (MDS-RS-SLD)	1	1 or 2	BM <5%, PB <1%, *without Auer rods*
MDS-RS with multilineage dysplasia (MDS-RS-MLD)	2 or 3	1–3	BM <5%, PB <1%, *without Auer rods*
MDS with del(5q)	1–3	1 or 2	BM <5%, PB <1%, *without Auer rods*
MDS with excess blasts (MDS-EB)			
MDS-EB1	0–3	1–3	BM 5–9% or PB 2–4%, *without Auer rods*
MDS-EB2	0–3	1–3	BM 10–19% or PB 5–19% or Auer rods
MDS, unclassifiable (MDS-U)			
With 1% blood blasts [3]	1–3	1–3	BM <5%, PB = 1%, *without Auer rods*
With single lineage dysplasia and pancytopenia	1	3	BM <5%, PB <1%, *without Auer rods*
Based on defining cytogenetic abnormality	0	1–3	BM <5%, PB <1%, *without Auer rods*

Typing

Morphology and Cytochemistry

MDS are typically characterized by a hyper- or normocellular bone marrow, which is in marked contrast to the cytopenia in the peripheral blood. The ineffective hematopoiesis is a defining characteristic of MDS. However, about 30% of patients with refractory anemia present with a hypocellular bone marrow, a so-called hypoplastic MDS. The morphology of cells in the peripheral blood in pediatric patients may show signs of dysplasia with dysgranulopoiesis, dyserythropoiesis, or dysmegakaryopoiesis (Figs. 35.18, 35.19, and 35.20).

Patients with refractory anemia frequently show disturbances in the maturation of megaloblasts of the erythropoiesis. This feature can be detected by macrocytosis in the peripheral blood. Occasionally, micromegakaryocytes are found in the periphery. More often, hypogranulated granulocytes and defects in the nuclear morphology, Pelger-Huët anomalies, or a dysplastic thrombopoiesis with micromegakaryocytes are observed.

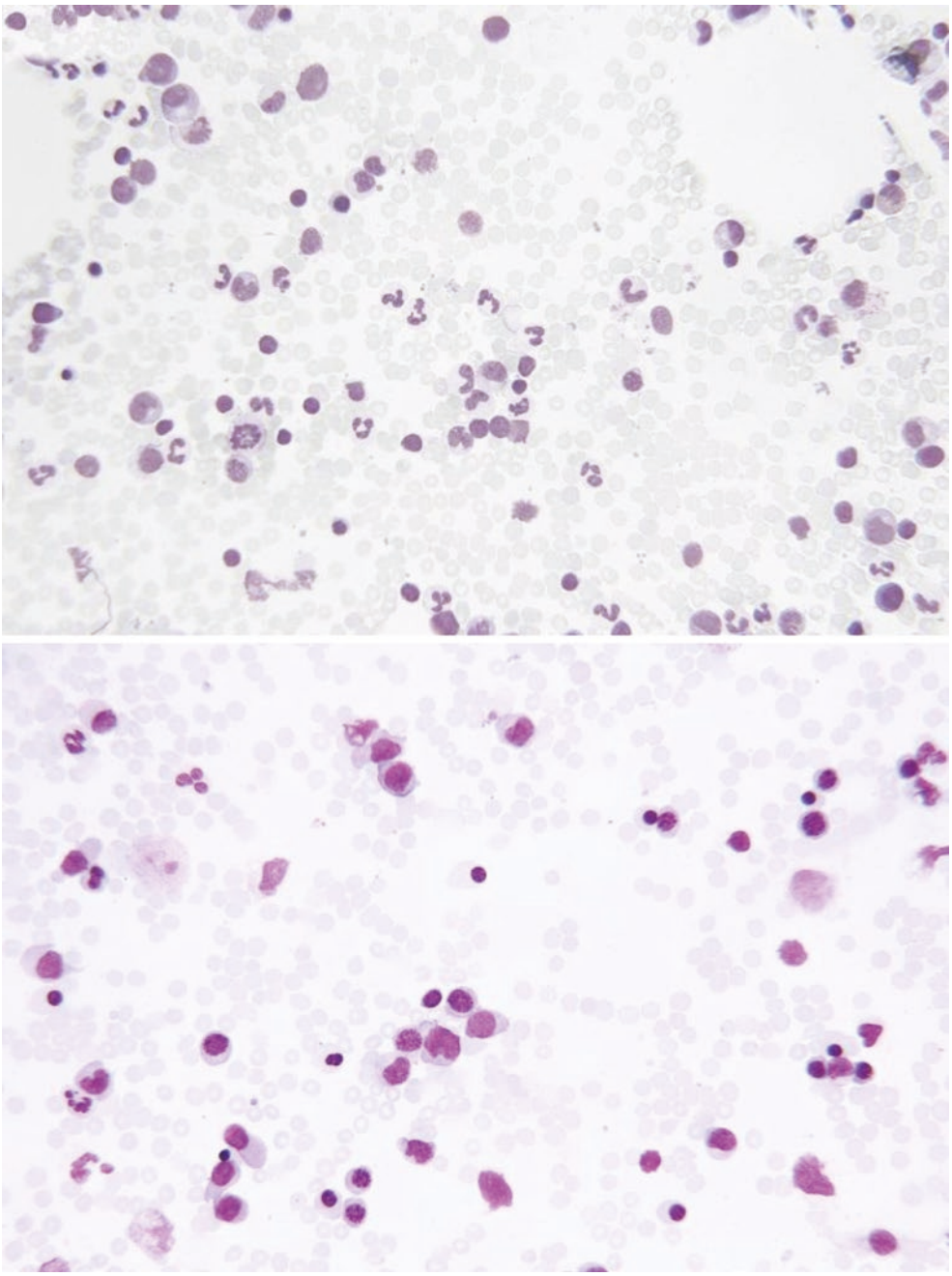

Fig. 35.18 Myelodysplastic syndromes (MDS) with signs of dysplasia of the granulopoiesis. Images show hypo- or agranulation of cytoplasm of neutrophil granulocytes as well as the dyserythropoiesis with macrocytic/megaloblastic changes

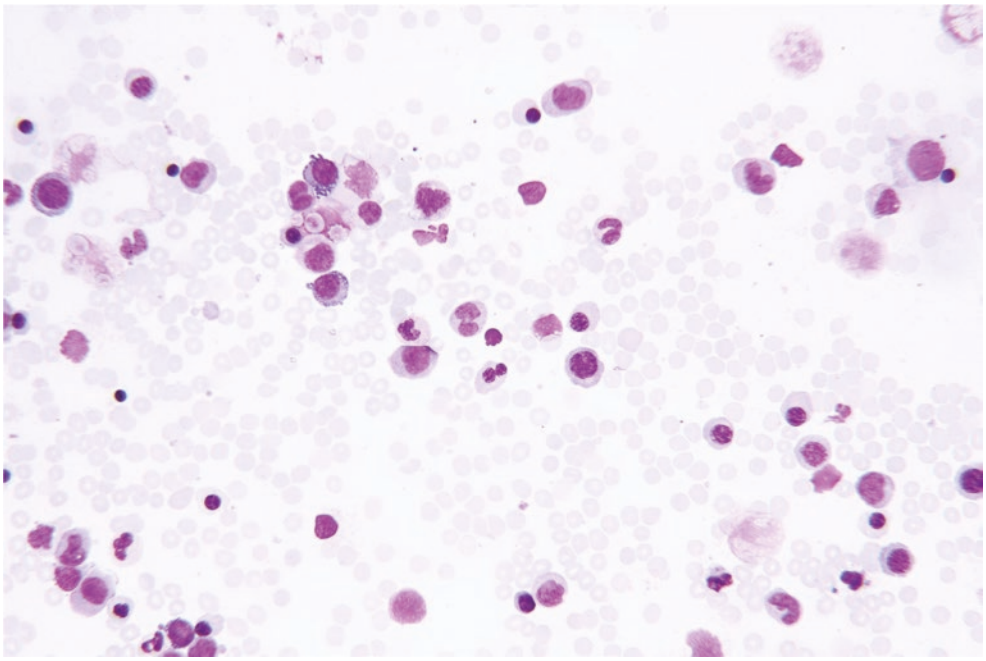

Fig. 35.19 Myelodysplastic syndromes (MDS) with signs of dysplasia of the erythropoiesis with multiple nuclei

Immune Phenotyping

During the initial evaluation of MDS, flow cytometric analysis should be considered to characterize blasts and detect abnormal lymphoid populations (e.g., increased hematogones). For example, a flow cytometric panel with anti-CD19, anti-CD33, anti-CD34, and anti-CD45 antibodies could be helpful. The European LeukemiaNet Working Group has identified four major abnormal constellations: the increased percentage of CD117+ precursor cells, a heterogeneous and low CD36 and CD71 expression, and an abnormal CD71/CD235 expression pattern [51]. For diagnosis and risk stratification of MDS, blasts should be further morphologically characterized. A more comprehensive antibody panel can be used with conspicuous subtypes (i.e., myeloid or lymphoid subtypes).

Cytogenetic Typing

Approximately half of pediatric patients with MDS present with a cytogenetic aberration in the bone marrow at the time of diagnosis. MDS generally comprise clonal aneuploidies and most commonly a loss of sections of chromosome 7, usually in the form of a complete monosomy or deletion of the long arm (del(7q)). Trisomy 8, trisomy 21, or complex karyotypes occur less frequently. A deletion of the long arm of chromosome 5 that is common in adult patients and associated with a typical clinical picture (also called 5-q-syndrome) is only rarely observed in pediatric patients. Until now, a clear correlation of a chromosomal aberration and a specific progression of the disease could not be detected. MDS-specific mutations are tet2, runx1, asxl1, sf3b1, srsf2, tp53, u2af1, dnmt3a, zrsr2, ezh2, nras, and kras.

Clinical Symptoms

MDS symptoms are mainly caused by the varying forms of cytopenia. In general, pediatric patients present with pale skin, fatigue, and performance reduction as a sign of anemia. Furthermore, hemorrhagic diathesis occurs as a result of a thrombocytopenia or functional disorders of the platelets and repeated fever conditions usually caused by bacterial infection. It is not uncommon that MDS are diagnosed randomly within regular pediatric checkups.

Therapy

The karyotype, peripheral blood counts, and bone marrow cellularity determine the therapy options in refractory cytopenia (MDS-RC). HSCT generally within 3 months after diagnosis is recommended in patients with monosomy 7, 7q-karyotype, or other complex karyotypes. A watch-and-wait strategy is possible for patients with ANC >1000/μL and no need for transfusion. In patients with ANC <1000/μL or a need for transfusion requiring therapy, some benefit from an immunosuppressive therapy with prednisone, cyclosporine A (CSA), and anti-thymocyte globulin (ATG). The responsive patients usually have a hypocellular bone marrow and normal karyotype or trisomy 8 only. A treatment response can be expected within 3–6 months. Usually, a partial response can be achieved. For patients with immunosuppression who do not respond to the therapy within the first 120 days, an unrelated donor should be searched, and HCT should be performed as soon as possible. Immunosuppression

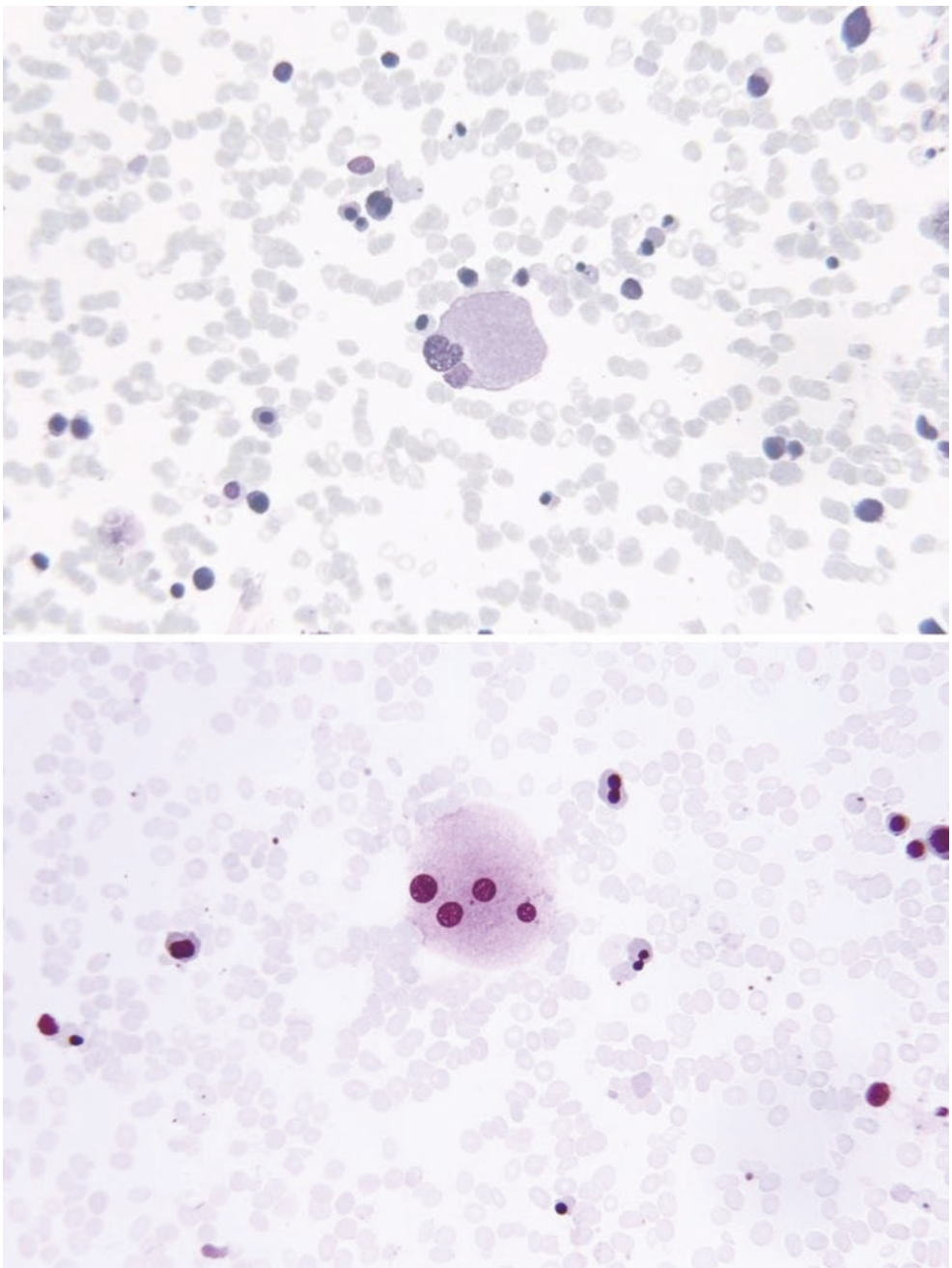

Fig. 35.20 Myelodysplastic syndromes (MDS) with signs of dysplasia of the thrombopoiesis. Images show a round, non-lobulated megakaryocyte and separated single nuclei

is not indicated for patients with normocellular or hypercellular bone marrow. HCT in these patients should be performed after a myeloablative conditioning regimen. During MDS with increasing blast counts (MDS-EB-I and EB-II), HCT is the appropriate treatment.

Hematopoietic Stem Cell Transplantation

Allogeneic HCT is the only curative treatment option for patients with MDS and increasing blast counts (MDS-EB-I and EB-II). The conditioning regimen should include administration of three alkylating agents that are independent from the cell cycle to act against resting stem cells aim to reduce relapses after transplantation and MDS-EB-I or MDS-EB-II. Since the relapse risk during refractory anemia is extremely low, it is recommended that the HCT is performed as soon as possible and before a possible progression to a higher disease stage with increased blast counts. Pediatric patients with a secondary MDS (7–18% of the MDS patients) after radio- or chemotherapy experience a fast progression and a poor prognosis after HCT. An intensive chemotherapy regimen is necessary before conditioning if there is an additional extramedullary infestation (e.g., skin infiltrations).

Relapse

The treatment options after a relapse after allogeneic HCT are limited and often accompanied by an unfavorable prognosis. Without an active therapy, the median survival time ranges between 3 and 4 months. The success of a therapy depends on the time lag between the initial allogeneic HSCT and the time of the occurrence of the relapse. Patients with a long-lasting complete remission after allogeneic HCT usually have better survival chances. The routinely used treatment options are a re-transplantation, the infusion of donor lymphocytes (DLI), or chemotherapy. The isolated administration of cytoreductive substances as a salvage therapy is not sufficient to achieve a permanent complete remission after a post-HCT relapse in MDS patients. Although remission rates of >40% are sometimes achieved, the remission is only persisting using a consolidation therapy with DLI or re-transplantation. Favorable prognostic factors for a re-transplantation are a young patient age, a complete remission of at least 6–12 months after initial allogeneic HCT, as well as a complete remission prior to the repeated allogeneic HSCT.

Long-Term Effects and Follow-Up

An allogeneic HCT is still accompanied with severe acute side effects and long-term effects. They are based on the high-dose chemotherapy as well as the transplantation itself: about 10% of patients develop a chronic GvHD affecting different organs and organ systems, most commonly the skin, the liver, and the gastrointestinal tract. The therapy can additionally cause damages of the lungs, the heart, the kidneys, the nervous system, the bone marrow, or the muscles. The endocrine system of children is particularly susceptible to be affected by the chemotherapy, whereby it can be partially or completely impaired. A hypofunction of the thyroid is often observed. Growth retardations (disturbances of the release of growth hormones) and retardations of the puberty (impairment of the sex hormone production) regularly occur. Therefore, regular and long-term follow-up care is especially important for pediatric patients after HCT. It includes regular clinical and laboratory analyses of the patient and a hormone substitution, if necessary. The chemotherapy before the HCT often leads to a permanent infertility and an increased risk for the development of a secondary malignant disease.

Juvenile Myelomonocytic Leukemia (JMML)

Definition

JMML is characterized by an excessive production of monocytes, and in some patients, immature blasts can be detected in the peripheral blood and the bone marrow. These cells infiltrate other organs and the organ system in which they are normally detected in only small numbers. Consequently, the affected patients generally present with hepatosplenomegaly. Frequently, the patients also experience invasions of these cells in other organs such as the lungs or the intestine. JMML is referred to as a mixed form of MDS and myeloproliferative neoplasms (MPN).

Incidence

The JMML is a rare disease in early childhood with an incidence of about 1–2 new cases per 100,000 children a year. The median age of patients at the time of diagnosis is 2 years. Boys are affected twice as frequently than girls. In over 15% of cases, JMML occurs in children with a congenital disorder, especially with neurofibromatosis type 1 (NF1) or the Noonan syndrome.

Prognosis

Unfavorable prognostic factors for the overall survival are low thrombocyte counts and a patient age under 2 years. A monosomy 7 does not affect the prognosis, but it causes characteristic differences to a JMML without monosomy 7.

Pathogenesis

JMML is caused by an excessive proliferation of myelomonocytic cells. An in vitro characteristic of JMML is an excessive endogenous production of the cytokine interleukin (IL) 1, granulocyte-macrophage colony-stimulating factor (GM-CSF), and TNF-α by monocytes. Furthermore, there is a hypersensitivity of the progenitor cells toward GM-CSF. Together, these characteristics lead to an apparently spontaneous proliferation of the progenitor cells of non-monocyte-depleted cell cultures. In 15–30% of cases, oncogenic RAS mutations cause a pathologic activation of the RAS/MAPK signaling cascade.

Diagnosis

The most important diagnostic criterion of JMML is an absolute monocytosis with monocyte counts of >1000/μL. The leukocyte count of most JMML patients is moderately increased. In 25% of patients, the leukocyte counts ranges between 50,000 and 100,000/μL.

Differential Diagnosis

Children with virus infections or leukocyte adhesion defects can present with similar clinical symptoms of a JMML. For differential diagnosis, the detection of a hypersensitivity of

myeloid progenitor cells toward GM-CSF is important. The cause of this hypersensitivity is not a mutation in the gene for the GM-CSF receptor but rather disturbances in the intracellular signal transduction in the course of RAS activation. Point mutations on the RAS gene that induce a constitutive activation of the RAS/MAPK signaling are detected in up to 25% of JMML patients.

Typing

Morphology and Cytochemistry

The bone marrow of JMML patients is normo- to hypercellular. Frequently, the megakaryocyte count is reduced and a granulocytic hyperplasia can be detected. Monocytosis in the bone marrow is less pronounced than in the peripheral blood. In 10% of patients, the blast percentage in the bone marrow exceeds 10%. The blood smear shows high amounts of blasts, lymphocytes, and monocytes as well as a granulocytopenia. In the bone marrow, a hyperplasia of the erythropoiesis and the granulopoiesis as well as an absence of megakaryocytes can be determined. Immature monocytic cells are prominent (Figs. 35.21 and 35.22).

Immune Phenotyping

There are now specific JMML antibodies. The immunohistochemically flow cytometry antibody staining of myelomonocytic components includes monoclonal antibodies against CD33, CD68, and myeloperoxidase.

Cytogenetic Typing

Depending on the specific mutations, different forms of the JMML can be defined:

- Acute myelomonocytic leukemia, somatic form with mutations of MLLT11 at 1q21.3
- Juvenile myelomonocytic leukemia, somatic form with mutations of ARHGAP26 at 5q31.3 or of PTPN11 at 12q24.13

In 75% of patients, mutations of NF1, NRAS, KRAS, or PTPN11 can be found. Losses of sections of chromosome 7 – usually as a complete monosomy or deletions of the long arm (del(7q)) – are the most frequent aberrations in JMML (25–30% of cases).

Clinical Symptoms

At the time of diagnosis, most important clinical signs are pale skin, lymphadenopathy, an expansive abdomen caused by the hepatosplenomegaly, petechiae, fever, and coughing. One-third of patients have skin infiltrates, mostly in the form of a maculopapular exanthema.

Therapy

Without proper therapy, JMML is fatal in 90% of cases. JMML hardly responds to chemotherapy or radiotherapy. A promising therapeutic option is HCT. Relapses occur in 35–50% of patients after HCT, on average within 3.5 months. Some of these patients can be healed by re-transplantation. In the posttransplant phase, the immunosuppressive GvHD prophylaxis should be ended early. The regenerating new immune system is thus possible to eliminate remaining leukemia cells of the patients (GvL reaction). Children with

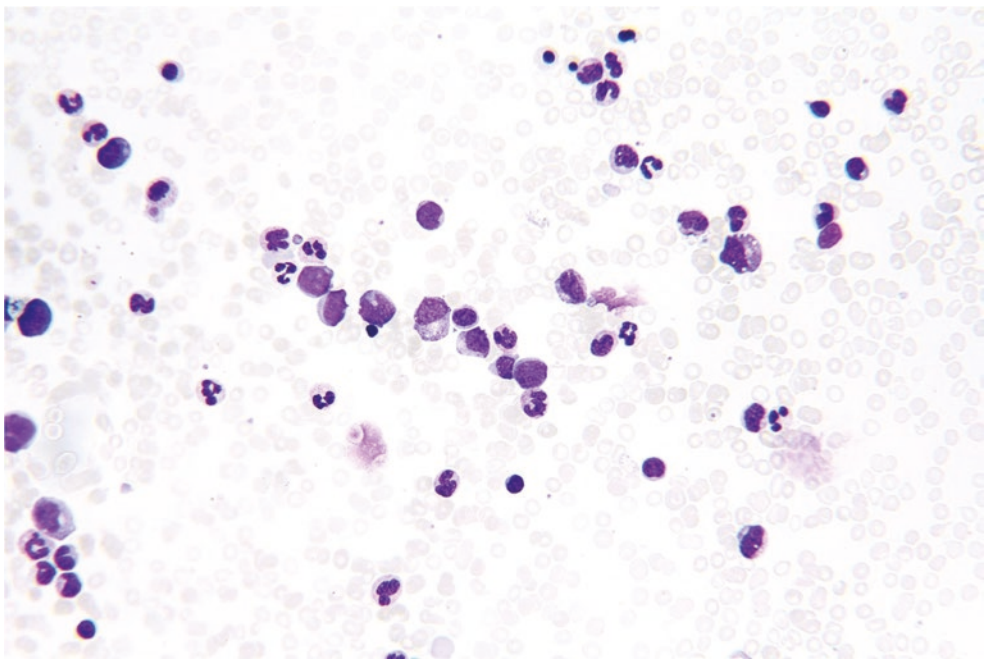

Fig. 35.21 Juvenile myelomonocytic leukemia (JMML). The blood smear shows high amounts of blasts, lymphocytes, and monocytes as well as a granulocytopenia and myelomonocytic blasts

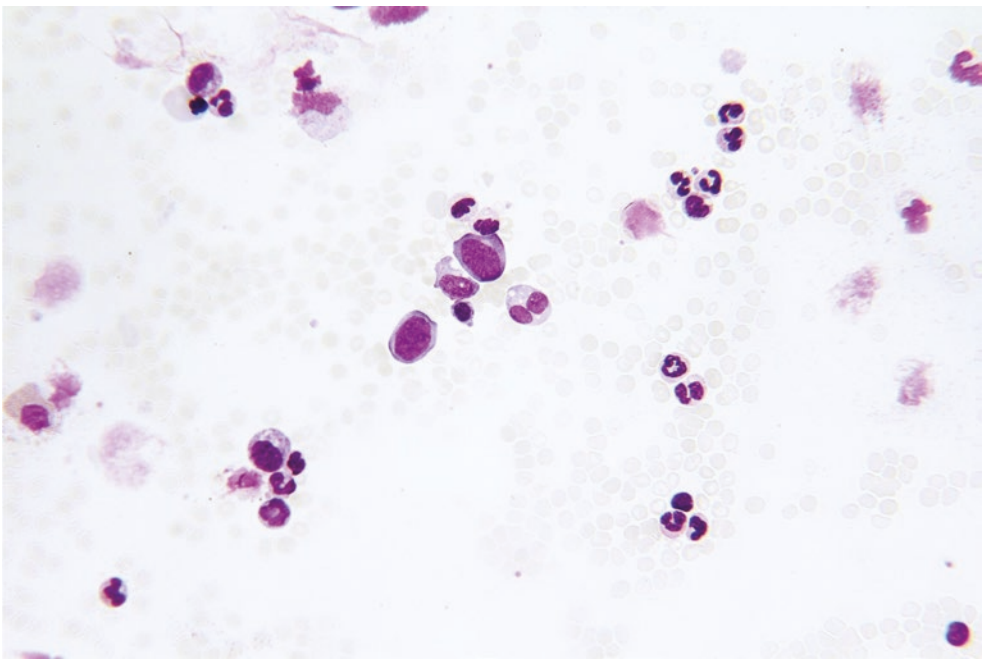

Fig. 35.22 Juvenile myelomonocytic leukemia (JMML). The bone marrow with a hyperplasia of the erythropoiesis and the granulopoiesis as well as an absence of megakaryocytes can be determined. Immature monocytic cells are prominent

JMML and Noonan syndrome should only be initially observed, since these patients regularly experience a spontaneous remission.

Relapse

If a relapse occurs after HCT, a re-transplantation has a healing chance of about 30%. A JMML relapse can be identified by chimerism analysis and in some patients with specific mutations by an MRD analysis.

Long-Term Effects and Follow-Up

Long-term follow-up after JMML especially includes the detection of transplant-related complications and long-term effects, similar to MDS.

Chronic Myeloproliferative Diseases

Chronic myeloproliferative diseases of children and adolescents include the CML, the essential thrombocythemia (ET), the primary myelofibrosis (PMF), and the polycythemia vera (PV). These clonal diseases – which derive from hematopoietic stem cells – are characterized by an autonomous proliferation of one or more cell lines of the hematopoiesis. These diseases may slowly proliferate and eventually transform into an acute and more aggressive form.

Chronic Myeloid Leukemia

Definition

CML is characterized by an increased hematopoiesis in the bone marrow, liver, and spleen. The CML is a common subtype of the MPN. During a CML, granulocyte counts increase uncontrollably and transfer from the bone marrow into the peripheral blood. The massive production of generally normal white blood cells then potentially causes several severe complications, such as blood clots or vessel occlusions.

Incidence

CML accounts for 2% of all leukemic diseases in pediatric patients younger than 15 years of age and for 9% in adolescents. With a male/female ratio of 1:8, females are affected significantly more often. The respective annual incidence rate is 1 and 2.2 cases in one million subjects in both age groups.

Prognosis

The 10-year survival rate of CML depends on the therapy used and ranges between 40% and 55%.

Pathogenesis

CML is a result of the malignant transformation of a multipotent hematopoietic stem cell. The consequence of this malignant transformation is an excessive myelopoiesis in the bone marrow, the liver, and the spleen. After an unknown period of time, the stable disease phase of the myelopoiesis develops to a phase of a myeloid or lymphatic blast crisis with a bone marrow insufficiency. The pathogenic mecha-

nism is based on genetic alterations of the hematopoietic cells in the bone marrow that may occur randomly, based on environmental factors, or may be favored through genetic disposition. In almost all patients, the so-called Philadelphia chromosome can be detected.

Diagnosis

In case of a suspected CML, a microscopic analysis of the peripheral blood should be conducted. In about 60–80% of patients, a mild anemia occurs. In contrast to other forms of leukemia, CML is always accompanied by a leukocytosis with leukocytes of all differentiation stages. In most cases, an increased percentage of basophil granulocytes can be found. In about 60% of cases, a thrombocytosis and in about 10% of cases a thrombocytopenia are observed. The cellular composition of the peripheral blood depends on the individual CML stage of the patient. Thus, the percentage of the blasts in the peripheral blood and the bone marrow can be used as an indicator for the current disease stage. A bone marrow study shows the proliferation of promyelocytes and myelocytes.

Differential Diagnosis

Leukemoid reactions have to be differentiated from the CML.

Typing

Morphology and Cytochemistry

In the peripheral blood, progenitors of the granulocytopoiesis (promyelocytes, myeloblasts, monocytes) can be found. These progenitors are usually cells with a large nucleus and little cytoplasm. Peroxidase-positive so-called Auer bodies (lysosomal degradation products) are found in the cytoplasm of granulocytes. Functioning, differentiated granulocytes are missing in the peripheral blood. In the bone marrow, a repression of the erythropoiesis and the megakaryopoiesis is seen. For the diagnosis of a CML, there must be about 25% blasts in the bone marrow or in the blood (Fig. 35.23).

Immune Phenotyping

Flow cytometric analyses during CML show myeloperoxidase-positive cells in about 80% of cases, HLA-DR in 20%, CD34 in 13%, CD117 in 43%, and CD13–33 in 79% of cases.

Cytogenetic Typing

For almost all CML patients, a specific genetic alteration – the Philadelphia chromosome – can be detected. Ph^1 is a causative factor for the development of the CML. This chromosome contains the mutated gene BCR-ABL, which is a fusion protein of the normal ABL gene and the normal BCR gene. Depending on the specific location of the insertion of ABL into the BCR region, different sizes of the fusion protein are determined. Characteristically, the 210 kDa fusion protein (p210) occurs in CML, and the 190 kDa fusion protein (p190) occurs in ALL. Additional chromosomal aberrations such as trisomy 8, isochromosome 17, trisomy 19, or a second Ph^1 can be detected in each metaphase.

Clinical Symptoms

Essentially three phases of the disease are distinguished: the chronic phase, the accelerated phase, and the blast phase or blast crisis. The majority of patients are in the chronic phase at the time of diagnosis, which can be maintained for several years by using modern targeted therapies. The chronic phase may take from 6 months up to 20 years. The accelerated phase describes the transformation of the chronic phase to the blast crisis. The blast crisis is similarly characterized to an acute leukemia. In contrast to the chronic phase, the blast crisis is usually therapy refractory, and it is generally fatal due to the progressing bone marrow insufficiency (bleeding, anemia, infections). The initiation of the disease takes several years and is mostly asymptomatic. In the course of the disease, general symptoms such as discomfort, fever, night sweats, performance reduction, and increased infection rates occur. In the advanced stage of the disease, splenomegaly occurs that may be accompanied with pressure or pain in the left upper abdomen and a tough, palpable spleen. In some patients, percussion or pressure tenderness can be provoked in the sternal area. During excessively increased leukocyte counts, spleen infarction or retinal veno-occlusion occurs occasionally, caused by leukemic blood clots.

Therapy

The treatment of CML is not standardized for pediatric patients and is usually adapted to the treatment guidelines for adult CML patients. A HCT in pediatric CML patients is currently used in patients with recurrent disease progression only. Children have a higher life expectancy and thus are subjected to a significantly longer tyrosine kinase inhibitor exposition than adult patients. Therefore, the major goal in the management of pediatric CML patients is to avoid a lifelong treatment with TKI. A possible solution is ending of the TKI therapy after a specific period of time with profound molecular remission. The aim of the therapy is an extensive normalization of the blood count in the sense of a remission. Therapeutic options include TKI (e.g., imatinib), interferons, chemotherapy with hydroxycarbamide (hydroxyurea), polychemotherapy, or allogeneic HCT as well as the combination of these therapies. HSCT remains the only curative treatment option.

Relapse

Treatment options for patients after therapy failure with imatinib include – besides the use of second-generation inhibitors (nilotinib or dasatinib) – the allogeneic HSCT after generally accepted criteria and recommendations.

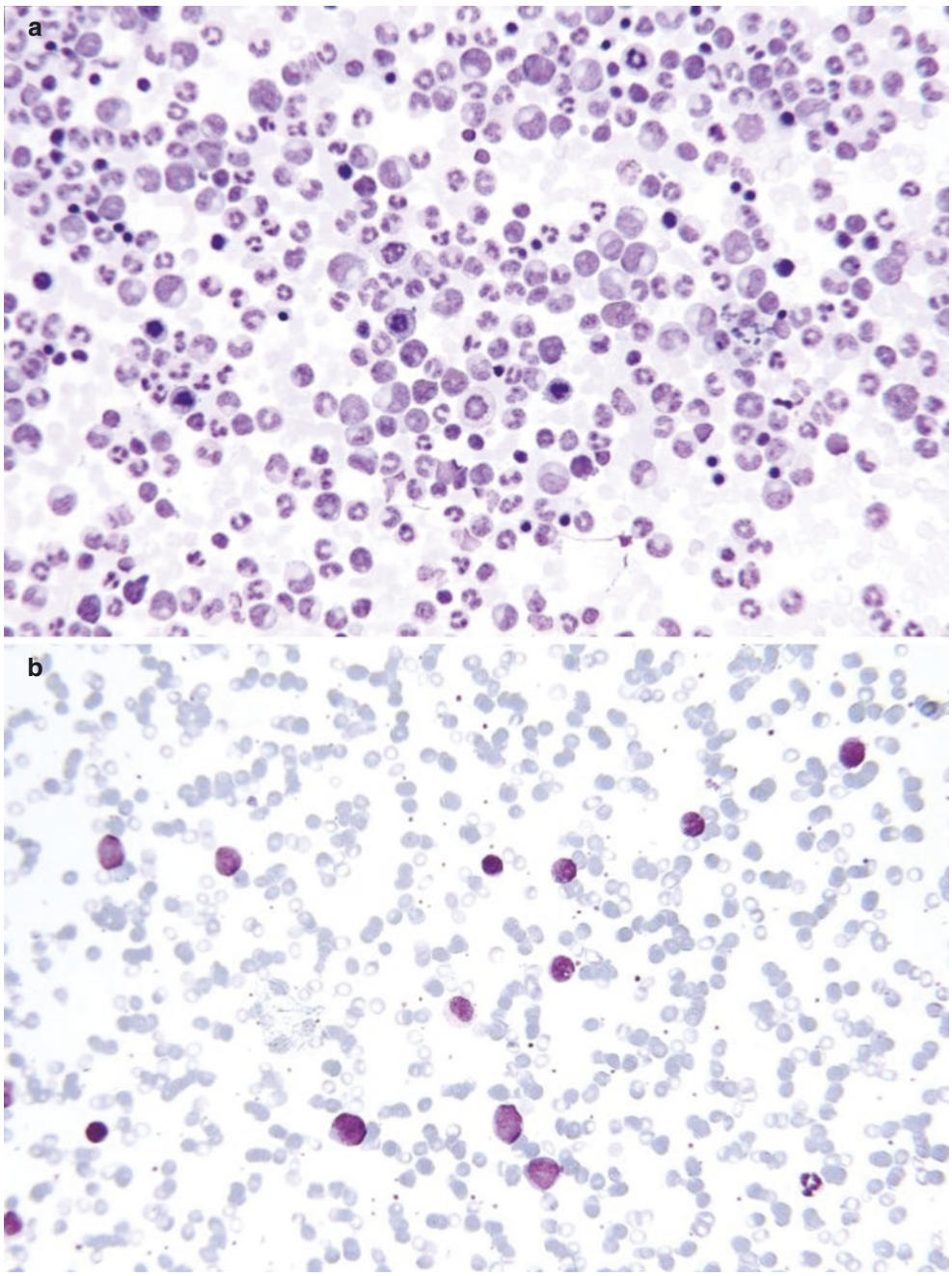

Fig. 35.23 Chronic myeloid leukemia (CML) with myeloid blasts. (**a**) shows the chronic CML phase with large amounts of eosinophil granulocytes, no blasts, a pronounced granulopoiesis, and few lymphocytes. (**b**) shows the acute CML phase with blast crisis

Long-Term Effects and Follow-Up

During the administration of imatinib, regular monitoring of the blood count every 3 to a maximum of 6 months is mandatory for the patients, since longtime use of TKI may cause severe long-term effects of the maturing child.

Essential Thrombocythemia (ET)

Definition

The essential (or primary) thrombocythemia belongs to the chronic myeloproliferative diseases (MPD) or neoplasms (MPN).

Prognosis

Risk factors for the development of thromboembolic and hemorrhagic complications in pediatric patients are anamnestically known thromboembolic complications or severe bleeding. Thrombocyte counts may increase >1,500,000/μL. Patients with ET have a normal life expectancy.

Pathogenesis

ET is a disease of the hematopoietic stem cell. It is characterized by a cytokine-independent increase of the megakaryopoiesis in the bone marrow and an increased release of thrombocytes in the peripheral blood. Through the discovery of specific disease-related genetic muta-

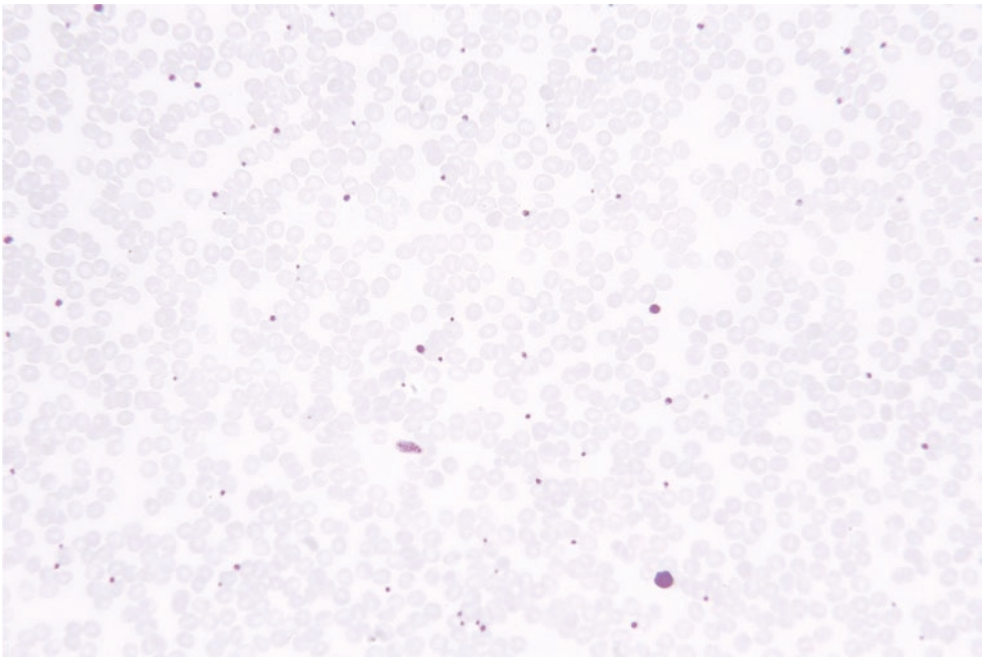

Fig. 35.24 Essential thrombocythemia (ET). ET with an increased proliferation of the megakaryocytic cell line and increase of the peripheral thrombocyte counts. In the bone marrow, the megakaryocytes are massively increased, often distinctly enlarged and arranged in loose groups. The nuclei are hyperlobulated (staghorn nuclear appearance)

tions, several molecular subtypes can currently be defined. The most frequent genetic aberrations are mutations of the *JAK2*^{V617F} (Janus kinase) (50% of cases in adults), CALR (calreticulin), and MPL (thrombopoietin receptor) genes.

Diagnosis

The diagnosis of ET is based on the WHO criteria, which are complemented by new findings in molecular genetic diagnostics:

1. Peripheral blood: thrombocyte counts persisting >450,000/µL.
2. Biochemical analysis: exclusion of a reactive thrombocytosis by CRP and ferritin determinations. Iron deficiency causes thrombocytosis.
3. Mutation analysis: JAK2^{V617F}, CALR, or MPL mutations.
4. With missing evidence of a CMPN typical mutation: exclusion of CML, MDS, or a different myeloid neoplasm.
5. Bone marrow for differential diagnosis: proliferation mostly of the megakaryocytic cell line with increased number and size of immature megakaryocytes, no fiber forming, no cloudlike megakaryocytic nuclei.

The diagnosis of an ET requires the presence of all five criteria.

Differential Diagnosis

For differential diagnosis, pre-analytical and analytical errors, a reactive thrombocytosis and myeloproliferative diseases should be excluded.

Typing

Morphology and Cytochemistry

ET is mainly characterized by an increased proliferation of the megakaryocytic cell line. The main symptom is the constant and usually slowly progressing increase of the peripheral thrombocyte counts. In the bone marrow, the megakaryocytes are massively increased, often distinctly enlarged and arranged in loose groups. The nuclei are hyperlobulated (staghorn nuclear appearance) (Fig. 35.24).

Cytogenetic Typing

A somatic mutation of JAK2^{V617F} is possible; mutations of the encoding gene for patients experience disorders of the microcirculation in fingers and toes (erythromelalgia; painful erythema with burning and swelling) or the brain (visual and speech disorders, migraine, vertigo). The most common and feared complication of ET is thrombosis in the venous and arterial system, such as the coronary arteries, the brain-supplying arteries, the large vessels of the upper abdomen (portal vein; liver, spleen, and mesenteric veins), and the veins of the lower extremities with consecutive pulmonary embolism. Paradoxically, hemorrhagic complications can occur in the presence of thrombosis is some patients due to abnormal platelet function.

Therapy

An antithrombotic prophylaxis (e.g., acetylsalicylic acid) is not established and controversially discussed, as well as a cytostatic therapy. The symptoms of the disease have to be carefully balanced against the potential side effects of the medication. Anagrelide – a quinazoline derivative and phos-

phodiesterase inhibitor – efficiently reduces the thrombocyte cell counts. It has a favorable side effect profile and does not essentially affect other myeloid elements. The mechanism of action seems to be mainly based on the effects on the megakaryopoiesis.

Long-Term Effects and Follow-Up

The ET can develop into a myelofibrosis. Regular examinations including clinical symptoms and blood testing have to be performed, as well as annual sonographic examinations of the abdomen (measurement of the spleen in three planes). Follow-up analyses of the bone marrow for detecting a rarely observed development of leukemia or myelofibrosis are indicated depending on the FU analyses (blood screening, spleen size) and are useful with suspected acceleration of the disease progression.

Primary Myelofibrosis (PMF)

Definition

PMF belongs to the chronic MPN. The bone marrow fibrosis occurs in clonal hematologic diseases (in children frequently in AML-M7). In combination with malignomas (i.e., bone metastases in patients with solid tumors, CML, Hodgkin lymphoma, and non-Hodgkin lymphoma), primary or secondary myelofibrosis can precede for weeks to months.

Incidence

PMF is very rare in children. Girls are affected more often than boys. A familial predisposition of PMF has been reported.

Prognosis

The 10-year survival rate of PMF is 20%. Disease progression varies individually.

Pathogenesis

PMF is a heterogenic disease. It develops from a hematopoietic stem cell. Genetic aberrations can occur in the JAK2, CAL, and/or the MPL gene.

Diagnosis

Due to the bone marrow fibrosis, a bone marrow aspiration fails as a "dry tap" (punctio sicca). Thus, a bone marrow biopsy needs to be carried out. In half of cases, a normo- to hypercellularity can be seen with an accentuated atypical thrombopoiesis. Splenomegaly-related symptoms, anemia and constitutional problems (fatigue, fever, bone pain, night sweat, and weight loss), thromboembolic complications, disturbances of the microcirculation, and bleeding can occur during PMF. The basic diagnostics contain the differential blood count, reticulocytes, LDH, ferritin, uric acid, prothrombin time, PTT, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-GT, alkaline phosphatase, bilirubin, Coombs test, and haptoglobin. For differential diagnosis with a suspected systemic mastocytosis, the serum tryptase can be determined. Genetic mutations of $JAK2^{V517F}$, CALR, and MPL should be identified.

Differential Diagnosis

For differential diagnosis, other chronic myeloproliferative diseases such as ET, PCV, and CML have to be excluded. Furthermore, primary (idiopathic) forms in which a secondary bone marrow develops caused by other chronic myeloproliferative diseases have to be differentiated. Bone marrow fibrosis can also occur in the progression of autoimmune disorders (collagenosis), MDS, or acute myelofibrosis during megakaryocytic leukemia (FAB-M7).

Typing

Morphology and Cytochemistry

There are cytogenetic anomalies of the erythrocytes [anisopoikilocytosis, erythroblasts in the peripheral blood, dacrocytes (tear drop cells)], and Pelger-Huët cells (two lobed neutrophils). Average-sized to huge megakaryocytes arranged in clusters and with cloudlike, bulbar, and often hyperchromic nuclei are dominant. In the further progression of the disease, hypercellularity is more dominant with a weakly to strongly pronounced bone marrow fibrosis. A leukoerythroblastosis is possible. A pancytopenia is then increasingly developing (Fig. 35.25).

Cytogenetic Typing

Cytogenetic aberrations frequently occur, e.g., as trisomy 8, trisomy 9, del(20q), del(13q), del(12p), or aberration on chromosomes 1 and 7. Mutations of $JAK2^{V617F}$, CALR, or MPL can occur.

Clinical Symptoms

The osteomyelofibrosis evolves insidiously and is characterized by collagen depositions in the bone marrow with increases of the spongiosa up to a total repression of the marrow with extramedullary hematopoiesis and progressive hepatosplenomegaly, accompanied by a portal hypertension. During the initial stage, the PMF is mostly asymptomatic. First signs of PMF are often detected within routine blood screenings as alterations in the blood counts (mostly thrombocytopenia and/or anemia). As the course of the disease progresses, the increasing fibrosis a repression of the normal hematopoiesis leads to symptoms of an ineffective hematopoiesis (anemia, thrombocytopenia, leukocytopenia, LDH increase), general symptoms (performance reduction, fever, night sweat, loss of appetite, weight reduction), as well as impairments by the extramedullary hematopoiesis (splenomegaly, hepatomegaly, bone pain). Clinical problems associ-

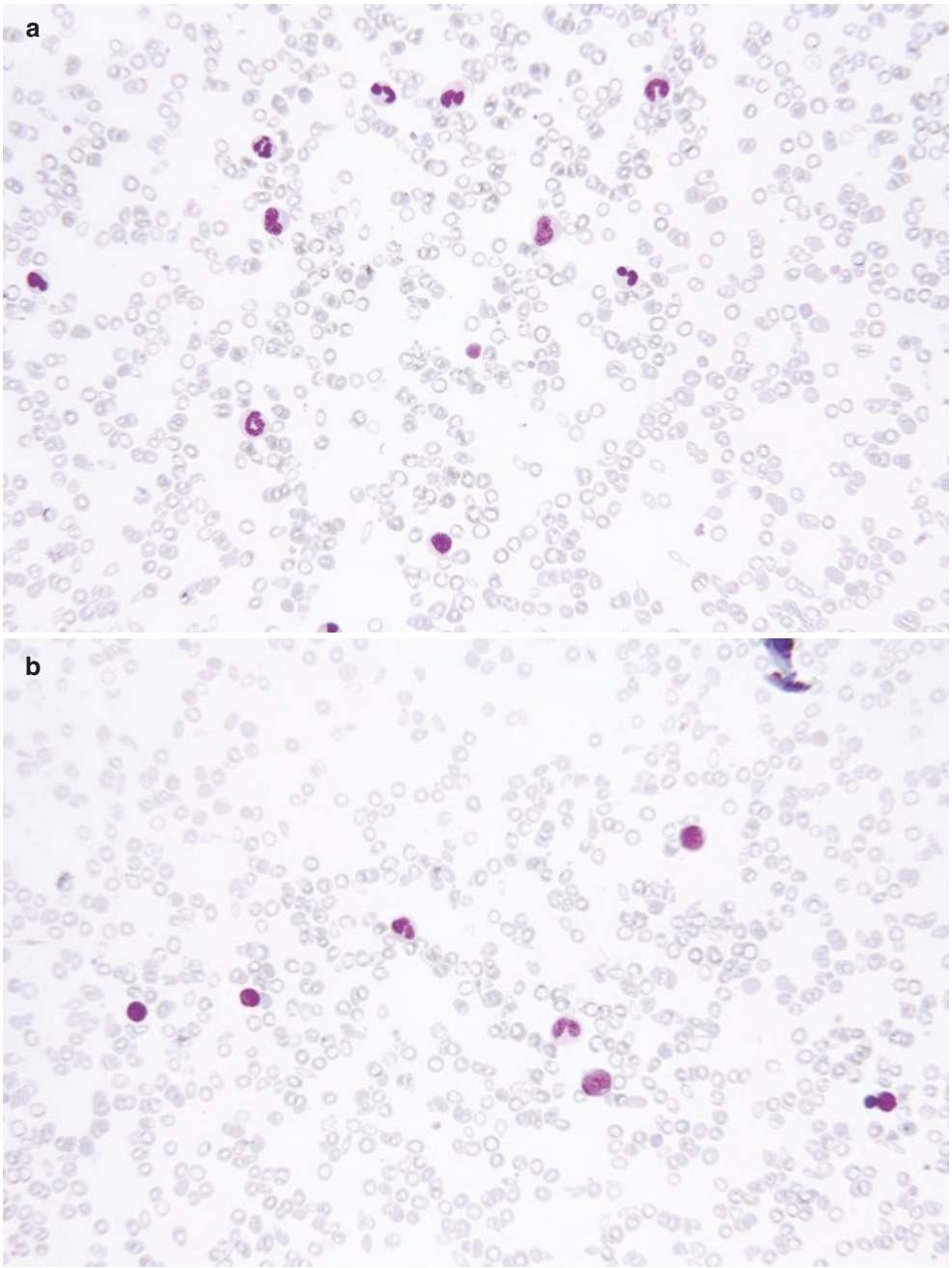

Fig. 35.25 Primary myelofibrosis (PMF). (**a**) shows megathrombocytes, an increased cell density with expansion of dysplastic and atypically distributed polymorphic megakaryocytes. (**b**) shows the preliminary stage of the granulopoiesis (myeloid blasts) and erythropoiesis with left shift and dysplasia

ated with the disorder that include thromboembolic complications at atypical sites (e.g., thrombosis of the portal vein, Budd-Chiari syndrome) can occur.

Therapy

A standard therapy for PMF does not exist; however, allogeneic HCT seems to comprise the best curative chances. HCT should be indicated within the 1st years after the initial diagnosis. The results of an HCT during an acceleration or blast phase are unfavorable. Thus, the allogeneic HCT should be carried out before reaching these phases. The drug therapy does not stop the progression of the disease. Therefore, the therapy indication during missing symptoms is rather cautious (watch-and-wait strategy). The oral JAK1/2 inhibitor ruxolitinib offers an effective and well-tolerated drug therapy for the treatment of PMF, mainly influencing the disease-related symptoms and the splenomegaly.

Long-Term Effects and Follow-Up

Clinical examinations such as determination of the spleen size, blood screenings including differential blood counts, and clinical chemistry should be carried out on a regular basis. FU analyses of the bone marrow for detecting a rare development of an acute leukemia, acceleration of the PMF,

or the increase of the myelofibrosis depend on the individual disease progression. If there are signs of a progression of the PMF (e.g., increasing anemia or thrombocytopenia, blasts in the peripheral blood), FU examination of the bone marrow should be considered. A quantitative FU monitoring of mutated JAK2 alleles can be helpful for therapy decisions in some cases. An annual sonographic examination of the upper abdomen is useful.

Polycythemia Vera (PV)

Definition

The PV is a rare myeloproliferative disease characterized by an increase of all three blood cell lines (especially erythrocytes, but also thrombocytes and leukocytes) in the peripheral blood.

Incidence

The PV is very rare in children.

Prognosis

The PV comprises an especially high risk when it remained undetected. Without proper treatment, the mean survival is only a few years. However, patients can have a normal life expectancy when the disease is observed continuously and treated correctly, if necessary.

Pathogenesis

The etiology of PV remains unclear. It is supposed that ionizing irradiation and chemical stressors (e.g., benzene) are causative for PV. According to pathophysiology, three different subtypes are classified: PV or primary PV, secondary PV, and relative PV. In about 98% of cases with clinical PV signs, mutations of the JAK2 gene are detectable. JAK2 mutations are not specific for PV, although JAK tyrosine kinases have an important function in the regulation of the proliferation of hematopoietic cells. By binding of specific cytokines (e.g., erythropoietin (EPO), TPO, G-CSF, GM-CSF) on surface receptors, the intracellular JAK2 proteins are phosphorylated and thereby activated. This leads to an activation of the transcription of signal molecules that activate the proliferation of hematopoietic cells. The $JAK2^{V517F}$ mutation and other mutations of the JAK2 (e.g., mutations in the JAK2 exon) cause a constitutive activation that is independent of the receptor binding of cytokines, leading to an uncontrolled and unregulated proliferation of hematopoietic cells. PV is dominated by an increased, EPO-independent proliferation of the erythropoiesis.

Diagnosis

PV can be accompanied by head pressure, vertigo, itching, arterial or venous thromboembolism, disturbances of the microcirculation, bleeding, hepatosplenomegaly, hypertension, and signs of a heart or pulmonary disease. Blood screenings include differential blood counts, BSG or CRP, LDH, ferritin, PT, aPTT, AST, ALT, γ-GT, alkaline phosphatase, bilirubin, uric acid, EPO in the serum, and an arterial blood gas analysis. Furthermore, sonography of the abdomen, chest X-ray, electrocardiography (ECG), echocardiography, and pulmonary function testing should be carried out. If the clinical and laboratory chemical results hint at a secondary (reactive) erythrocytosis, the relevant primary disease is diagnosed and treated internally. A PV is highly probable when an erythrocytosis and a simultaneous leukocytosis and/or thrombocytosis and/or splenomegaly as well as a left shift and/or isolated erythroblasts in the blood smear are detected. Aspirational cytology and histology with serum levels of iron and EPO should be analyzed. When the screening for a $JAK2^{V517F}$ mutation is negative, JAK2 exon 12 mutations and Philadelphia chromosome should be identified. In adults, 90% of polycythemia vera is associated with the JAK2 polymorphism.

Differential Diagnosis

PV has to be differentiated from secondary (reactive) erythrocytosis and the very rare non-MPN forms of the primary erythrocytosis. The following erythrocytosis types have to be considered:

1. Transient erythrocytosis with simultaneous increase of hematocrit and hemoglobin concentrations during severe exsiccation
2. Acquired secondary erythrocytosis based on arterial hypoxia during chronic heart and pulmonary diseases
3. Inherited causes of erythrocytes (rare and partially sporadic forms): erythropoietin receptor mutations, gain-in-function mutations of erythropoietin or its receptor, hemoglobinopathy with increased oxygen affinity, 2,3-DPG deficiency (e.g., 2,3-DPG mutase deficiency), and defects of the hemoglobin production during normal oxygen affinity of the hemoglobin (heterozygous beta thalassemia, alpha thalassemia minor, mild forms of iron deficiency anemia; Hb, hematocrit, and mean erythrocyte volume are decreased in these cases)

Typing

Morphology and Cytochemistry

A stimulation of all three hematopoietic systems (panmyelosis) with repression of the adipocytes, hyperlobulated nuclei of the megakaryocytes, a hypocellular bone marrow, and an intramedullary iron decrease often occurs (Fig. 35.26).

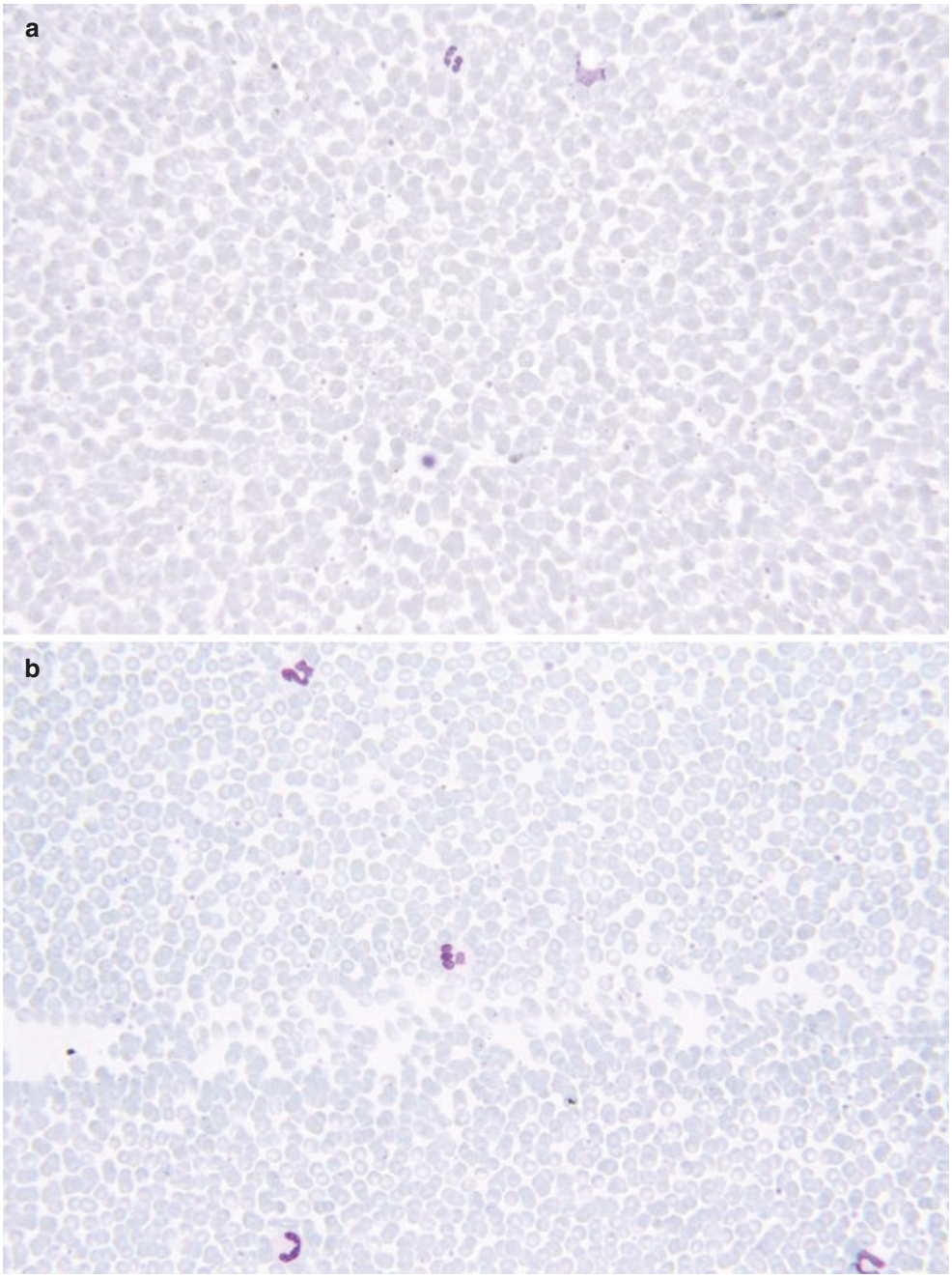

Fig. 35.26 Polycythemia vera (PV). PV is often characterized by repression of the adipocytes, hyperlobulated nuclei of the megakaryocytes, a hypocellular bone marrow, and an intramedullary iron decrease. (**a**) shows megathrombocytes. (**b**) Image shows hypersegmented granulocytes

Cytogenetic Typing

In 80% of the cases, a mutation of the JAK2 gene can be found.

Clinical Symptoms

The diagnostic criteria of PV include an increased erythrocyte volume, splenomegaly, thrombocytosis, leukocytosis, increased alkaline phosphatase, and increased vitamin B12 or unsaturated vitamin B12 binding capacity. The erythrocytosis is predominant and determines the clinical picture. Results of the increased viscosity of the blood by increases of the hematocrit are symptomatic disturbances of the microcirculation and an increase risk for thromboembolic complications. Two different clinical stages of the PV can be classified: a chronic phase with increase erythrocyte production and erythrocytosis that can last up to 20 years and a progressive late phase that is accompanied by secondary myelofibrosis (post-PV myelofibrosis) with extramedullary hematopoiesis and increasing splenomegaly and/or the development of a myelodysplasia or AML. Further symptoms are headache, weakness, weight loss, pruritus, and vertigo.

Therapy

Due to the leukemogenic potential, cytotoxic agents and radiotherapy with phosphorus compounds should not be used for cytoreduction in pediatric patients. A regular phlebotomy is often sufficient to control the hematocrit. Hydroxyurea (if thrombocytes are >1000,000/μL), interferon (IFN)-α or IFN-γ, and anagrelide are used in adult patients to reduce the cell counts and are also effective in pediatric patients. The therapy depends on the symptoms, the erythrocyte counts, and the arterial oxygen status (oxygen saturation, oxygen saturation curve). A standard therapy regimen does not exist for pediatric patients. However, the hematocrit should be maintained <45% to avoid thrombohemorrhagic complications. Erythrocytes also can be depleted by apheresis, but phlebotomy is the safest route.

Long-Term Effects and Follow-Up

Regular clinical and laboratory examinations should be carried out. Occasionally, phlebotomy-free phases occur in which an extension of the FU intervals is possible up to 3 months. Morphological analyses of the blood (blood smear), by a left shift, detection of erythroblasts and/or tear-shaped erythrocytes, and the development of an MDS or leukemia can be identified. The diagnostic proof can be given by a bone marrow puncture. FU analyses of the bone marrow for the detection of rare developments of an AML or myelofibrosis are indicated with signs of a disease progression (e.g., increasing splenomegaly or changes in the blood counts). A quantitative FU monitoring of mutated JAK2 alleles can be helpful for therapy decisions in some cases.

Summary

During the past three decades, the therapy of leukemia has undergone a substantial transformation. Due to the consistently developing research and the progress being made with new therapeutic options, we currently have the opportunity to cure more patients than ever before. Furthermore, pediatric and adolescent patients that cannot be cured may at least benefit from a prolonged survival and an improved quality of life. If the patients do not respond to the initial therapy, an alternative treatment strategy can be pursued in most of the cases. Many of the new treatment forms are still highly specific and are thus only suitable for specific cases; however, these strategies are constantly evolving. Pediatric neoplasms comprise specific cytogenetic and molecular biological properties that are different from adult cancers. This chapter gives a comprehensive overview of the current knowledge on pediatric hematological disorders, including standard therapy regimen; molecular biological, genetic, and morphological typing; supportive care; acute complications; and novel targeted therapy options.

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Transfusion Medicine

36

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Introduction

The field of blood banking and transfusion medicine incorporates elements of blood donation and collection, blood component manufacture, pretransfusion testing including serology and molecular methods, blood transfusion therapy, and clinical consultation.

Blood and blood components are regulated by the Food and Drug Administration (FDA) as "biological products" as defined in section 351 of the PHS Act, approved July 01, 1944 (42 U.S.C. 262) and in addition are regulated as drugs under the Federal Food, Drug, and Cosmetic Act of June 25, 1938 (21 U.S.C. 301–392). The Code of Federal Regulations, or CFR, contains the regulations pertaining to blood purity, potency, and efficacy. Blood collection primarily takes place in blood centers that supply blood to hospital transfusion services. Blood centers recruit donors and collect, process, manufacture, store, and distribute blood components. FDA has established five layers of safety to protect the blood supply, which comprise donor screening, donor testing, product testing, quarantining, and inspections to monitor and investigate aberrancies.

This book chapter reflects the views of the author and should not be construed to represent FDA's views or policies.

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Transfusion services are responsible for conducting pretransfusion testing, selecting and issuing components in coordination with clinicians, and providing medical consultation. Very few hospital transfusion services at present perform collections. The majority of hospital transfusion services are supplied with their blood product inventory by collaborating regional blood centers.

Transfusion services that are certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA'88) or have met equivalent requirements and perform only basic component preparation are not required to register with the FDA. Professional organizations such as the AABB (formerly known as the American Association of Blood Banks), the College of American Pathologists (CAP), and The Joint Commission provide accreditation standards for transfusion services and conduct voluntary inspections. Registered and licensed facilities are inspected by the FDA every 2 years.

Donors

Blood and blood components for transfusion are collected from volunteer donors. The majority of donations are allogeneic donations, intended for use in other patients. However, a small minority are autologous collections, provided by individual patients for their own use in advance of a scheduled surgical procedure to compensate for anticipated perioperative blood loss. The efficacy of autologous collections in improving patient outcomes, however, is unproven. Conversely perioperative autologous donation may have unintended adverse consequences, such as adverse donation reactions or increased preoperative anemia, causing the need for additional allogeneic transfusions [1].

To be eligible to donate, donors must be in good health and apparently free from transfusion-transmitted infections. Allogeneic donors complete a Donor Health Questionnaire (DHQ) prepared by the AABB Donor History Task Force and recognized by the FDA as an acceptable mechanism for collecting reliable blood donor history information, consis-

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tent with FDA requirements and regulations. To be eligible, donors have successfully answered health-related questions assessing any past or present illnesses and risk factors and satisfy minimum physiologic criteria. Selected current eligibility criteria for donations of whole blood are listed in Table 36.1. Additional criteria apply to donors of apheresis (a procedure using semiautomated centrifugation devices that separate cellular and plasma components of whole blood) products. Donors are instructed to contact the donor center should any complications arise post-donation, or they become aware of any reason their blood may not be safe.

Donated blood is tested for the donor's ABO group and Rh type, including testing for the presence of weak D antigen. In addition, a sample from each donation for allogeneic use is tested by FDA-licensed tests for antibodies to human immunodeficiency virus (anti-HIV-1/2), hepatitis C virus (anti-HCV), and human T-cell lymphotropic virus (anti-HTLV-I/II) and for hepatitis B core antigen (anti-HBc) and for hepatitis B surface antigen (HBsAg). Licensed nucleic acid tests (NAT) for hepatitis B virus (HBV) deoxyribonucleic acid (DNA), HCV ribonucleic acid (RNA), HIV-1 RNA, and West Nile virus (WNV) RNA are also performed. At the time of this writing, all blood components except for source plasma (plasma that is intended for fractionation into albumin, immune globulins, etc.) are currently tested by a nucleic acid test for Zika virus [2]. In addition, a serologic test for syphilis is performed, and donors must test negative by a licensed test for antibodies to *Trypanosoma cruzi* either on the current donation or at least one previous donation. Some units are tested for antibodies against cytomegalovirus (CMV).

Table 36.1 Donor eligibility criteria, 21 CFR 630.10 and 630.15

Donors must have no factors indicating an increased risk for a transfusion-transmitted infection (TTI)
Must not have been institutionalized for >72 h consecutively in a correctional facility in the past 12 months (where there may be an increased risk of contracting TTI)
Must be free from illness
Must not have traveled to or resided in an area endemic for a TTI including malaria
Must not be pregnant at time of donation or within 6 weeks prior to donation
Must not be under the influence of drugs or alcohol
Must not be a recipient of xenotransplantation
Temperature must be ≤37.5 °C (99.5 °F)
Females must have hemoglobin ≥12.5 g/dL or hematocrit ≥38%[a]; males must have hemoglobin ≥13 g/dL or hematocrit ≥39%
Autologous donors must have hemoglobin ≥11 g/dL or hematocrit ≥33%
Systolic blood pressure ≤180 mm of mercury, ≥90 mm of mercury; diastolic blood pressure ≤100 mm of mercury, ≥50 mm of mercury[b]
Pulse must be between 50 and 100 beats per minute and regular[c]
Weight must be ≥50 kg (110 lbs)
Skin must be free of infection, inflammation, and lesions; arms must be free of scars or lesions indicative of intravenous drug injection
The interval between whole blood donations must be at least 8 weeks (or if donating the equivalent of 2 red cell units by apheresis, must be at least 16 weeks)

[a]Blood may be collected from females with hemoglobin between 12.0 and 12.5 g/dL provided the procedure does not adversely affect the health of the donor and has been approved by the FDA

[b]Donors with blood pressures outside these ranges may be permitted to donate only when the responsible physician (RP) has determined and documented that the health of the donors would not be adversely affected by donating

[c]Donors with heart rates outside these ranges may be permitted to donate only when the RP has determined and documented that the health of the donors would not be adversely affected by donating

Blood Bank Policies and Procedures

Pretransfusion Testing

Pretransfusion testing is performed in the blood bank with the purpose of ensuring that blood components are serologically compatible with the intended recipient. Routine pretransfusion testing includes ABO and Rh typing and an antibody screen. ABO typing tests for red blood cell A and B antigens and corresponding anti-B or anti-A in the serum, while Rh typing tests for the Rh(D) antigen. The antibody screen is an indirect antiglobulin test using commercial red blood cells from two to three type O donors whose collective red blood cell phenotype is known to be positive for the most common clinically significant red cell antigens. A positive antibody screen indicates clinically significant antibodies may be present and further evaluation to determine the identification of the antibody is required. Antibody identification is performed using commercial kits of panels of typed donor cells. A crossmatch is performed as the final step to ensure pretransfusion compatibility. If the antibody screen is negative, the primary purpose of the crossmatch is to detect ABO incompatibility and can be performed by direct agglutination, or the "immediate spin." In the presence of a positive antibody screen, the antiglobulin technique is required.

Transfusion Administration and Monitoring

After issue, blood units must be carefully matched with the recipient using at least two patient identifiers (full name and date of birth or medical record number) on the blood unit and the patient wristband. Misidentification of the blood unit or the patient can result in catastrophic potentially fatal hemolytic reactions. Therefore prior to administration of a blood transfusion, both the patient and the unit must be identified accurately.

Blood transfusions are overseen by the clinician caring for the patient in conjunction with the transfusion medicine

service physician and the blood bank. A physician's order is necessary to dispense blood products. All blood components must be administered through a filter, usually 170–260 μm to filter clots and particles. Blood components should be administered with normal saline. RBCs should not be given with 5% dextrose and hyper- or hypotonic saline due to the potential for hemolysis.

Peripheral access with an 18-gauge needle is usually sufficient, whereas smaller lumen catheters may cause hemolysis. The patient should be monitored closely for at least the first 15 min of the blood transfusion and routinely assessed thereafter until completion of the blood product infusion. Blood transfusions are usually given over a time period of 30–120 min in clinically stable adults. In stable pediatric patients, blood components are usually administered at a rate of 10 mL/kg over 2–3 h.

In urgent situations when time does not permit for antibody identification, selection of type O red blood cells and group AB plasma is considered safe practice. Rh-negative red cells are preferred to prevent sensitization to Rh(D), in particular for females of childbearing age. However, in an emergent situation, Rh-positive red cells may be necessary and are often given to males and females >50 years of age. As soon as time permits for adequate compatibility testing, the blood bank transitions to providing type-specific, crossmatched blood. Communication between the blood bank and the treating clinician is critical to ensure timely and appropriate delivery of blood products without delay or error.

Blood Utilization/Transfusion Committees

The number of transfusions administered has sharply decreased over the last several years. In 2015, a total of 11,114,000 whole blood and RBC units were transfused, a 15.7% decline from 2013. This followed a 4.4% decline of whole blood and RBC transfusions from 2011 [3]. The decrease is due largely to increased recognition of the adverse effects of transfusion and the resulting implementation in many facilities of patient blood management (PBM) programs. The goal of PBM programs is to decrease transfusions that are not indicated and to reduce unnecessary blood utilization [4]. PBM programs monitor blood utilization and can intervene directly if a particular clinician is observed to transfuse at higher rates than his/her colleagues in a similar situation. Transfusion committees are a multidisciplinary group variably comprised of a representative from the blood bank (a transfusion medicine physician), anesthesia, surgery, obstetrics, internal medicine and nursing, and others. The role of the Transfusion Medicine Committee is to meet to review transfusion cases, oversee and review blood utilization, and implement cross-disciplinary procedures and protocols. Methods include education on evidence-based transfusion guidelines, decision support in the computerized provider order entry system, and distribution of provider-specific reports showing comparison to peers for guideline compliance, among other metrics.

Whole Blood and Blood Components

21 CFR 606.3(a) defines blood as a fluid containing dissolved and suspended elements which was collected from the vascular system of a human, while a blood product consists of whole blood, plasma, serum, or any product derived from human whole blood (21 CFR 607.3(b)). Whole blood donations are often manufactured into components in order to target blood delivery according to specific patient requirements for red blood cells, plasma, or platelets. Benefits of component therapy include maximizing individual donations to serve the specific indications of multiple recipients, thereby reducing waste while supplying each patient with the precise component needed.

Whole Blood (WB)

(a) Overview
Whole blood collections consist of approximately 450–500 mL of whole blood collected into anticoagulant solutions such as citrate phosphate dextrose (CPD) and CP2D and stored at 1–6 °C for 21 days or collected into citrate phosphate dextrose adenine-1 (CPDA-1) and stored for up to 35 days. Whole blood units are prepared aseptically in a ratio of 14 mL of anticoagulant-preservative solution per 100 mL of whole blood intended for collection.

(b) Indications
Whole blood transfusions are indicated for the treatment of symptomatic anemia or critical deficits of oxygen-carrying capacity. The optimal use of whole blood is in the treatment of patients who are experiencing traumatic hemorrhagic shock and immediately life-threatening injuries.

(c) Evidence
Although infrequently available today in civilian blood banks, whole blood use has a long history in the military. It was first introduced in World War I and used in the US military from 1917 until the Vietnam War era when crystalloids and colloids replaced blood as the primary initial resuscitative measure for hemorrhagic shock [5, 6]. However recent combat experience has revived interest in the use of group O whole blood, driven by US Army data indicating improved or comparable survival compared to blood component therapy [7]. Additionally it is increasingly recognized that overuse of crystalloids and colloids in the initial treatment of shock and acute blood loss can

contribute to worsening coagulopathy and decrease oxygen delivery, and balanced resuscitation with blood is the optimal approach for severe bleeding [8]. Therefore, component therapy to mimic "reconstituted whole blood" is a common approach in hemostatic resuscitation in trauma.

Logistically however component therapy is a challenge on the battlefield. Platelets, which have a maximum shelf life of 5 days, are frequently unavailable in the combat setting. Consequently, the use of whole blood began increasing in large combat hospitals in the early 2000s. As evidence of the benefit of whole blood mounted, in 2014 the Tactical Combat Casualty Care Committee recommended that group O whole blood should be the preferred resuscitative product for patients with traumatic hemorrhagic shock over all other blood product combinations and fluids [9].

(d) Contraindications/Adverse Events
Circulatory overload is a risk from whole blood administration in patients not in need of volume replacement. Normovolemic patients with chronic anemia should not be given whole blood but treated with RBC (i.e., plasma reduced) components. Residual white blood cells (WBCs) can potentially cause transfusion-associated graft-versus-host disease (TA-GVHD), a rare but almost always fatal reaction, particularly in immunosuppressed recipients (see Section J.b.ii on TA-GVHD below). In military settings, whole blood is occasionally transfused before time allows for testing of infectious agents to be performed and thus can pose a higher risk of transfusion-transmitted infection.

(e) Administration
Whole blood is administered through a 170- to 260-micron filter. The rate of administration can be tailored for the patient, but the entirety of the unit should be transfused within 4 h. Whole blood stored longer than 24 h contains few viable platelets or granulocytes. In an adult, one unit of whole blood will increase the hemoglobin level by approximately 1 g/dL or the hematocrit by about 3–4%. Whole blood must be ABO identical with the recipient, except in military situations where use of group O whole blood with low-titer anti-A/anti-B antibodies is under investigation.

Red Blood Cells

(a) Overview
RBCs can be prepared by centrifugation or sedimentation of whole blood to remove the majority of the plasma. Alternatively, RBC components can be obtained by apheresis methods. Apheresis collection of RBCs allows collection of a co-component or double (2-unit) red cell collection. RBCs are stored at 1–6 °C in a variety of anticoagulant solutions. The anticoagulants vary in composition and determine the shelf life of the product: 21 days with anticoagulant citrate dextrose-A (ACD-A), CPD, or CP2D and 35 days with CPDA-1. Following plasma removal, red cells have a hematocrit of 65–80% and a volume between 225 and 350 mL. If RBCs are stored in additive solution (AS, i.e., solutions containing additional nutrients and normal saline), the hematocrit is 55–65% with a volume between 300 and 400 mL. AS extends the shelf life of RBCs to 42 days. The average storage duration of RBCs in the United States ranges from 19 to 22 days [10].

(b) Indications
RBCs are indicated to treat symptomatic anemia in patients who require an increase in oxygen-carrying capacity and red cell mass. They are also indicated for red cell exchange transfusion. Although professional guidelines recommend transfusion requirements should be based on individual patient needs and not a predetermined hemoglobin or hematocrit value, practices continue to vary [11–14].

(c) Evidence
An ongoing issue has focused on potential adverse effects of older stored blood due to a red blood cell storage lesion. RBCs stored for longer time periods may have impaired oxygen delivery ability due to factors such as increased red cell membrane rigidity, decreased nitric oxide metabolism, and accumulation of inflammatory mediators. However, multiple randomized clinical trials (RCTs) published in recent years evaluating the effect of red blood cell transfusions in various patient populations failed to show adverse effects of blood stored up to 35 days [15–20] [21]. In consideration of the new data, recently released clinical practice guidelines from the AABB on red blood cell transfusion thresholds and storage provide recommendations for both clinicians caring for hemodynamically stable hospitalized adult patients needing RBC transfusions and for transfusion medicine services responsible for storing and providing RBCs [22].

Regarding RBC transfusions, the guidelines recommend a restrictive RBC transfusion threshold. The recommendation states that transfusion is not indicated until the hemoglobin level is 7 g/dL for hospitalized patients who are hemodynamically stable, including critically ill patients, as opposed to a liberal threshold of 10 g/dL. For patients undergoing orthopedic surgery or cardiac surgery and those with preexisting cardiovascular disease, the guidelines recommend a restrictive transfusion threshold of 8 g/dL. The recommendation was based on multiple RCTs performed in various clinical settings in more than 12,000 patients. The recommendation does not apply to patients with acute coronary syndrome, severe thrombocytopenia (patients treated for hematological or oncological disorders who are at risk of bleeding), and chronic transfusion-dependent anemia due to lack of sufficient evidence provided by RCTs.

The second recommendation is that patients, including neonates, should receive RBC units selected at any point within their licensed dating period (standard issue)

Table 36.2 Duration of red blood cell storage and clinical outcome

Reference	Study population	RBC storage duration in days	Primary outcome
INFORM Heddle et al. [18]	General population of hospitalized patients Total patients in primary analysis, *N* = 20,858 Fresh, *N* = 6936 Older, *N* = 13,922	Mean (SD) Fresh – 13 (7.6) Older – 23.6 (8.9)	Mortality Fresh – 9.1% Older – 8.7% Odds ratio 1.05 (95% CI* 0.95–1.16) *P* = 0.34
ABLE Lacroix et al. [19]	Critically ill adult ICU patients Total patients in primary analysis, *N* = 2412 Fresh, *N* = 1206 Standard, *N* = 1206	Mean (SD) Fresh – 6.1 (4.9) Standard – 22 (8.4)	Mortality Fresh – 37% Standard – 35.3% ARD† 1.7, (95% CI –2.1 to 5.5)
RECESS Steiner et al. [20]	Patients ≥12 years of age undergoing complex cardiac surgery Total patients in primary analysis, *N* = 1098 Fresh, *N* = 538 Standard, *N* = 560	Mean (SD) Fresh – 7.8 (4.8) Older – 28.3 (6.7)	Change in the 7-day multiple organ dysfunction score (MODS, range 0–24)‡ Fresh – 8.5 ± 3.6 Older – 8.7 ± 3.6 Estimated Treatment effect −0.2 (95% CI -0.6 to 0.3) *P* = 0.44
TOTAL Dhabangi et al. [15]	Children aged 6–60 months with malaria or sickle cell disease Total patients in primary analysis, *N* = 286 Fresh, *N* = 143 Standard, *N* = 143	Median (IQR) Fresh – 8 (7, 9) Older – 32 (30, 34)	Lactate level ≤3 mmol/L Fresh – 83/143 (58%) Older – 87/143 (61%) Between group difference 0.03 (95% CI, −0.07 to ∞) *P* = <0.001
ARIPI Fergusson et al. [16]	Premature infants with birth weights <1250 g in the neonatal ICU Total patients in primary analysis, *N* = 377 Fresh, *N* = 188 Standard, *N* = 189	Mean (SD) Fresh – 5.1 (2) Standard – 14.6 (8.3)	Composite measure of major neonatal morbidities § as well as death Fresh – 52.7% Standard – 52.9% Relative risk 1.01 (95% CI 0.90–1.12)
TRANSFUSE Cooper et al. [21]	Critically ill adult patients in the ICU Total patients in primary analysis N = 4919 Fresh, N = 2457 Standard, N = 2462	Mean (SD) Fresh – 11.8 (5.3) Standard – 22.4 (7.5)	90-day all-cause mortality Fresh – 24.8% Standard – 24.1% Odds ratio 1.04 (95% CI 0.91–1.18) *P* = 0.57

†Absolute risk difference, ‡Multiple organ dysfunction score, §Including necrotizing enterocolitis, retinopathy of prematurity, brochopulmonary dysplasia, intraventricular hemorrhage, and death

rather than limiting patients to transfusion of only fresh (storage length: <10 days) RBC units. The recommendation was made based on 13 trials including neonates and infants with very low birth weights and children and adults. The trials did not include patients undergoing massive or exchange transfusion, neonates, and children with renal disease at risk for hyperkalemia, intrauterine transfusions, or patients with hemoglobinopathies requiring chronic transfusion support. The trials compared fresher blood with standard-issue blood. The mean storage duration of the standard-issue RBCs in the primary trial of neonates was 14.6 days, while in the trials of adults, the mean storage duration was 28 days or less. Although no evidence was seen that transfusion of fresher RBCs is superior to standard-issue RBCs for risk of mortality, it is of note that no trials to date have addressed the question of blood at the end of storage duration (42 days) compared to fresher blood [22]. An overview of the study designs and results from select trials assessing duration of red cell storage and clinical outcomes is provided in Table 36.2.

(d) Contraindications/Adverse Events

Complications due to transfusion of red blood cells are notable for hemolytic transfusion reactions, both acute and delayed. They frequently are a cause of alloimmunization. Other risks include transfusion-associated circulatory overload (TACO) and iron overload. Red cells should not be used to treat anemias that can be treated with medications such as iron, vitamin B12, folic acid, or erythropoietin. However, another consideration is that the use of erythropoietin in certain populations, e.g., those with underlying malignancy, may lead to tumor progression [23]. Red cells should also not be administered primarily for volume expansion.

(e) Administration
RBCs must be administered through a 170- to 260-micron filter. The rate of administration can be adjusted according to the patient's tolerance; however products containing RBCs should not be kept at room temperature longer than 4 h. No medications or solutions may be added to or infused through the same tubing simultaneously with blood or blood components other than 0.9% Sodium Chloride Injection (USP) unless approved for this use by the FDA, and there is documentation that the additive is safe. The ABO group of all red cell-containing components must be compatible with ABO antibodies in the recipient's plasma. Unlike whole blood, RBCs need not be ABO identical. In an adult, one unit of RBCs will raise the hemoglobin level by approximately 1 g/dL or the hematocrit by about 3%. In a pediatric patient, 10 mL/kg will raise the hemoglobin level by 2–3 g/dL or the hematocrit by about 6%.

Plasma Components

(a) Overview
Plasma is the aqueous portion of blood comprising over half of total blood volume. Plasma contributes to the maintenance of intravascular volume and contains albumin, coagulation factors, fibrinolytic proteins, immunoglobulins, and other proteins. Plasma can be prepared by the separation of a whole blood collection or collected by apheresis. Plasma units contain on average 200–250 mL, but apheresis-derived units may have volumes ranging from 400 to 600 mL.

Following collection, plasma can be frozen and later thawed prior to use or maintained in the liquid state. Various formulations of both frozen and liquid plasma products exist. Fresh-frozen plasma (FFP) is separated from the RBCs or collected by apheresis and placed at ≤ −18 °C within 8 h of collection. Plasma frozen within 24 h after phlebotomy (FP24) is similarly prepared but can be placed at ≤ −18 °C at any time up to 24 h following collection. Plasma for transfusion is generally referred to as FFP, frozen within 8 h after phlebotomy; in practice, however the majority of plasma for transfusion is FP24, frozen within 24 h of phlebotomy. FFP and FP24 can generally be transfused interchangeably although FP24 is not indicated to treat deficiencies of labile coagulation factors, e.g., Factors V and VIII. An additional frozen plasma product is plasma frozen within 24 h held at room temperature up to 24 h (FP24RT24), which has indications comparable to FP24.

Plasma, Cryoprecipitate Reduced is prepared from whole blood-derived FFP after thawing and centrifugation and removal of the cryoprecipitate. The remaining plasma is deficient in fibrinogen, Factor VIII, Factor XIII, von Willebrand factor (vWF), cryoglobulin, and fibronectin. It must be refrozen at ≤ −18 °C within 24 h of thawing. Plasma, Cryoprecipitate Reduced is used for transfusion or plasma exchange in patients with thrombotic thrombocytopenic purpura (TTP), although it has not been demonstrated to improve outcomes versus FFP in TTP patients. It may be used to provide clotting factors other than fibrinogen, Factor VIII, Factor XIII, and vWF.

Liquid plasma products include thawed plasma and liquid plasma. Thawed plasma is an unlicensed liquid product stored at 1–6 °C with a shelf life up to 5 days after thawing. Hospitals using this product report reduced plasma product outdating. Thawed plasma serves as a source of nonlabile plasma proteins [24]. Liquid plasma is a product separated and infused no later than 5 days after the expiration date of the whole blood. It is stored at 1–6 °C and is indicated for the initial treatment of patients undergoing massive transfusion due to life-threatening trauma or blood loss who have clinically significant coagulation deficiencies.

(b) Indications
Plasma components supply plasma proteins to patients who have deficient or dysfunctional plasma proteins. Common indications for plasma transfusion include management of preoperative or bleeding patients who require replacement of multiple plasma coagulation factors (e.g., liver disease, disseminated intravascular coagulopathy (DIC)), patients undergoing massive transfusion who have clinically significant coagulation deficiencies due to dilutional coagulopathy from volume/blood replacement (see section on massive transfusion, below), rapid reversal of warfarin in a bleeding patient or emergent surgical situation, transfusion or plasma exchange in patients with TTP, management of patients with specific coagulation factor, or plasma protein deficiencies for which no targeted coagulation concentrates or recombinant products are available.

(c) Evidence
In 2010 the AABB published evidence-based practice guidelines for plasma transfusion [25] although made note of the fact that scientific evidence supporting many plasma transfusion practices is limited and/or weak. Despite evidence ranging largely from very low to moderate, the panel issued the following recommendations for plasma transfusion: trauma patients requiring massive transfusion and patients with warfarin anticoagulation-related intracranial hemorrhage. Due to insufficient evidence, the panel could not recommend in favor or against transfusion of plasma at a plasma/RBC ratio of 1:3 or higher ratios (e.g., 1:2 or 1:1, more plasma per RBC) in trauma patients during massive transfusion, transfusion of plasma for patients undergoing surgery in the absence

of massive transfusion, or transfusion of plasma to reverse warfarin in patients without intracranial hemorrhage. The panel recommended against plasma transfusion in other groups of patients (i.e., those not undergoing massive transfusion, surgery, bleeding, or excessive anticoagulation), such as patients with acute pancreatitis, organophosphate poisoning, or acetaminophen poisoning.

The British Society of Hematology recently released guidelines regarding the processing, selection, and use in specific populations of fresh frozen plasma and cryoprecipitate products. Importantly, the guidelines emphasize that there is no evidence to support the prophylactic use of FFP in non-bleeding patients with abnormal pre-procedure coagulation tests [26].

(d) Contraindications/Adverse Events
Plasma transfusions are not indicated when coagulopathy can be treated more effectively with specific therapy [27] and are not indicated as a primary means for volume expansion. Plasma transfusions should not be given to correct minor abnormalities in laboratory coagulation parameters as they correlate poorly with bleeding propensity. Transfusion-related acute lung injury (TRALI), discussed in more detail below, is a significant risk of plasma transfusion, although less than previously, since plasma is not obtained from female donors potentially sensitized against HLA antigens through pregnancy. Plasma can elicit allergic or anaphylactic reactions, particularly in IgA-deficient patients. Volume overload (or TACO, transfusion-associated circulatory overload) and transfusion-transmitted infection are additional plasma transfusion hazards.

(e) Administration
Frozen plasma products should be infused immediately after thawing or stored at 1–6 °C for up to 24 h. After 24 h, if collected in a functionally closed system, the product may be labeled as thawed plasma. As with other blood products, plasma must be administered through a filter. The volume transfused depends on the clinical situation and the underlying disease. For coagulation factor replacement, the dose is 10–20 mL/kg or 3–6 units in an adult. The expected increase in coagulation factors immediately following transfusion of the above dose is approximately 20%, although the actual increase is often lower owing to coagulation factor consumption and/or continuing hemorrhage. Monitoring of the patient's coagulation status following transfusion is important to guide further therapy. Coagulation function tests such as prothrombin time/international normalized ratio (PT/INR), activated partial thromboplastin time (aPTT), and specific factor assays should be monitored; however laboratory results do not consistently correlate with clinical status. Plasma products must be ABO compatible with the recipient's red cells.

Cryoprecipitated Components

(a) Overview
Cryoprecipitated antihemophilic factor (AHF) contains fibrinogen, Factor VIII, Factor XIII, vWF, and fibronectin. It is prepared by thawing whole blood-derived or apheresis FFP at 1–6 °C and removing the supernatant plasma. The cold-insoluble precipitate and 10–15 mL of plasma remain in the bag, which is then placed at −18 °C or colder within 1 h and has a shelf life of 1 year. Each unit of cryoprecipitated AHF should contain ≥80 IU of Factor VIII and ≥150 mg of fibrinogen in approximately 5–10 mL of plasma. With current manufacturing processes, the amounts of factor content per unit generally far exceed the specified requirements.

(b) Indications
Cryoprecipitate is indicated to treat congenital or acquired fibrinogen deficiency and can be used to treat Factor XIII deficiency when FFP cannot be used due to volume concerns and/or recombinant products are not available. It is often used in surgical settings to raise fibrinogen levels in patients with acquired coagulopathy with hemorrhage, such as trauma or obstetric hemorrhage [28, 29]. It can be used as second-line therapy to treat Factor VIII deficiency and vWD; however specific coagulation factor preparations should be used if available. Second-line use can be considered for adjunctive control of uremic bleeding if other methods have failed, such as desmopressin (DDAVP).

(c) Evidence
Cryoprecipitate was originally used in the treatment of Factor VIII deficiency in the 1950s [30]; however it is currently not often used for that purpose, due to the availability of specific coagulation factor preparations. It is licensed in some countries for the treatment of hypofibrinogenemia, to be administered at a fibrinogen threshold <100 mg/dL; however these recommendations are empiric [30]. In contrast, cryoprecipitate has been withdrawn in many European countries and replaced with commercial fibrinogen preparations due to safety concerns (e.g., pathogen transmission) [31]. FDA has licensed a fibrinogen concentrate (RiaSTAP), for patients with congenital fibrinogen deficiency including afibrinogenemia and hypofibrinogenemia, but it is not indicated for dysfibrinogenemia or other indications. Therefore, cryoprecipitate remains the FDA-licensed product for use in settings other than congenital deficiencies [32, 33].

(d) Contraindications/Adverse Event
Cryoprecipitate is not indicated in the treatment of Factor VIII deficiency or vWD when factor concentrates are available. It should not be used unless labora-

tory results indicate a specific hemostatic defect for which this product is indicated. In rare situations, infusion of large amounts of ABO-incompatible cryoprecipitate can cause hemolysis; smaller amounts can result in a positive direct antiglobulin test (DAT). The risk of transfusion-transmitted infection from a unit of cryoprecipitate is the same as that of FFP; however often cryoprecipitate is usually administered in pools of five units, increasing donor exposure and potentially increasing risk. It can also be implicated as a cause of TRALI.

(e) Administration
Before infusion, cryoprecipitate is thawed at 37 °C. It is administered through a 170- to 260-micron filter. Compatibility testing is not required; however, ABO-compatible products are preferable. Each unit is expected to increase fibrinogen in an average-sized adult by 5–10 mg/dL. Typically, five single units are pooled into one bag for a standard dose. In an actively bleeding patient or prior to surgery, cryoprecipitate should be given when fibrinogen falls below 100 mg/dL. Thawed cryoprecipitate should be kept at room temperature and transfused as soon as possible after thawing. The thawed product can be kept at room temperature for up to 6 h if pooled using an FDA-cleared sterile connecting device or for a single unit but must be transfused within 4 h if the container is entered without using an FDA-cleared sterile connecting device.

Platelet Components

(a) Overview
Platelets can be collected by apheresis or derived from units of whole blood. Whole blood-derived platelets must contain at least 5.5×10^{10} platelets per unit, whereas apheresis platelets are required to have a minimum of 3.0×10^{11} platelets per unit. In practice, these components typically exceed these requirements. Platelets have a shelf life of up to 5 days (or 7 days if an apheresis platelet in plasma component is tested within 24 h of transfusion with a bacterial detection test cleared by the FDA as a "safety measure") and are stored at room temperature (20–24 °C) with continual agitation. Platelets that are pooled using an open system must be transfused within 4 h of pooling. The pH must be maintained at ≥6.2 throughout storage.

(b) Indications
Platelet transfusions are indicated for patients with hypoproliferative thrombocytopenia, congenital or acquired platelet dysfunction who are bleeding or at high risk of bleeding, and in some surgical and invasive procedures.

(c) Evidence
In 2014 the AABB published evidence-based guidelines addressing the indications for platelet transfusions [34]. The guidelines strongly recommended prophylactic platelet transfusions to reduce the risk of spontaneous bleeding in adult hospitalized patients with therapy-induced hypoproliferative thrombocytopenia and a platelet count <10,000/μL. In addition, they provided weak recommendations for prophylactic platelet transfusion for elective central venous catheter placement at a platelet count <20,000/μL, elective diagnostic lumbar puncture at a platelet count <50,000/μL, and major elective nonneuraxial surgery with a platelet count <50,000/μL, as well as platelet transfusion for patients undergoing coronary artery bypass graft (CABG) with perioperative bleeding and thrombocytopenia and/or evidence of platelet dysfunction. The guidelines recommended against routine prophylactic platelet transfusion for non-thrombocytopenic patients having cardiac surgery with cardiopulmonary bypass, as perioperative platelet transfusions have been shown to independently predict adverse outcomes. However, if patients undergoing cardiopulmonary bypass show signs of perioperative bleeding, thrombocytopenia, or qualitative platelet abnormalities (which can be associated with exposure to the bypass circuit), the guidelines recommend consideration of platelet transfusion. Recently published guidelines from the American Society of Clinical Oncology addressing platelet transfusion for patients with cancer propose that pooled and apheresis platelets can be used interchangeably, as studies have demonstrated comparable hemostatic efficacy and safety with these products [35].

(d) Contraindications/Adverse Event
Platelet transfusion may not provide clinical benefit in cases of thrombocytopenia due to immune-mediated destruction of circulating platelets, such as ITP and TTP [36], or in situations of rapid platelet destruction such as DIC. Platelet transfusions are relatively contraindicated in such situations. In general, platelets should not be transfused if the platelet count is greater than 100,000/μL, unless there is known platelet dysfunction.

One of the main risks of platelet transfusion is sepsis due to bacterial contamination, discussed in further detail below. Other risks associated with platelet transfusion include allergic reactions, platelet alloimmunization, and febrile nonhemolytic reactions (FNHTRs).

(e) Administration
An adult platelet dose is considered to be the equivalent of one apheresis unit or a pool of 4–6 whole blood-derived units. An average platelet dose can increase the platelet count by 30,000–60,000/μl, although, as described below, lower recoveries are often seen owing to consumption

and/or continuing hemorrhage. In children, the average dose is 10 ml/kg. Failure to achieve an expected increment in platelet count can indicate refractoriness. Platelet refractoriness can be caused by a variety of conditions including bleeding, splenomegaly, DIC, fever, sepsis, drugs, and immunization to human leukocyte antigen (HLA) or platelet antibodies. Response to platelet transfusion can be assessed with the corrected count increment (CCI), determined by the following formula: CCI = [(post-tx plt ct)-(pre-tx plt ct) × BSA (m^2)]/(number of platelets transfused × 10^{11}), where post-tx plt ct = post-transfusion platelet count, pre-tx plt ct = pretransfusion platelet count, and BSA = body surface area in square meters. A CCI of <7500 from a sample drawn up to 1 h posttransfusion or a CCI of <4500 from a sample drawn 18–24 h posttransfusion suggests platelet refractoriness.

Granulocyte Components

(a) Overview
Granulocytes are prepared by cytapheresis from a single donor. Each unit contains $\geq 1.0 \times 10^{10}$ granulocytes as well as 20–50 mL of RBCs and variable amounts of lymphocytes and platelets. Corticosteroids are often administered to the donor to augment granulocyte yield. Granulocyte colony-stimulating factor (G-CSF) is a more potent option that can increase the granulocyte yield up to $4–8 \times 10^{10}$ granulocytes/bag. However due to logistical obstacles such as requiring a licensed healthcare professional for administration, and donor side effects including bone pain, myalgia, arthralgia, nausea/vomiting, and headache, G-CSF is not utilized at many facilities. Red cell sedimenting agents, e.g., hydroxyethyl starch (HES), are typically used in granulocyte collection. After suspension in plasma and addition of anticoagulant, the product volume ranges between 200 and 300 mL.

(b) Indications
Granulocytes are indicated for patients with non-resolving infections who are neutropenic (ANC <500/μL), unresponsive to appropriate therapy, have a reasonable chance of regaining function, and have been treated appropriately for at least 24–48 h. They can also be appropriate for patients with hereditary neutrophil function defects such as chronic granulomatous disease (CGD).

(c) Evidence
Granulocyte transfusions have been in use for many years, but efficacy has never been conclusively demonstrated. A 2015 phase III randomized controlled trial sponsored by the National Institutes of Health, the Resolving Infections in Neutropenia with Granulocytes (RING) study, evaluated the efficacy of G-CSF/dexamethasone-mobilized granulocyte transfusion in neutropenic patients on chemotherapy with infections [37]. No difference in clinical outcome was observed between the control and the treatment group, and a secondary analysis found statistically significant better results regarding clinical course and survival in subjects who received high-dose treatment ($\geq 0.6 \times 10^9$/kg), although the study was underpowered statistically. A recent review shows that despite conclusive evidence of clinical efficacy of granulocyte transfusion, largely due to low enrollment in clinical trials, such transfusions can play a critical role in supporting neutropenic patients with life-threatening bacterial infections [38].

(d) Contraindications/Adverse Events
Granulocytes should not be given in the absence of documented infection, neutropenia, and lack of response to an appropriate treatment regimen for an appropriate length of time. A trial of broad-spectrum antibiotics should have been implemented before granulocyte transfusion is considered. Granulocytes should also not be given if a patient does not have a reasonable chance of recovery. There is a risk of viral transmission, particularly cytomegalovirus (CMV). Therefore, if a CMV-seronegative patient is severely immunosuppressed, serious consideration should be given before administering CMV-seropositive granulocytes if CMV-seronegative granulocytes are not available. Pulmonary events are a significant complication, particularly with high-dose transfusions. Other transfusion reactions such as fever, chills, and allergic reactions are also associated with granulocyte transfusions.

(e) Administration
Granulocytes can be stored up to 24 h at 20–24 °C without agitation and however should be administered as soon as possible after collection to avoid deterioration of granulocyte function during short-term storage. The product should be transfused with a 170- to 260-micron filter. A leukocyte reduction filter however should not be used. Daily transfusions are often given; however for some patients every other day transfusions are sufficient. Components can be gamma-irradiated if the patient is at risk of TA-GVHD. Granulocytes must be ABO compatible due to the high number of red cells present.

Further Processing

(a) Leukocyte Reduction
The presence of leukocytes in blood and blood components can lead to adverse effects, including FNHTRs, HLA alloimmunization, and viral transmission. A unit of whole blood contains on average $\geq 1 \times 10^9$ white cells.

Leukocyte reduction has been implemented by the majority of blood establishments for all transfusions of RBCs and platelets in order to reduce the adverse effects of WBCs. Leukocyte reduction can be performed by in-process apheresis collection or filtration. It is often performed prestorage (soon after collection) but can also be done post-storage (at the time of transfusion, considered less robust that leukocyte reduction performed at the collection center under controlled conditions). A leukocyte-reduced RBC or apheresis platelet must have no more than 5×10^6 residual leukocytes/unit, and a leukocyte-reduced whole blood-derived platelet must have no more than 8.3×10^5 residual leukocytes/unit.

Leukocyte reduction has proven benefit in reducing FNHTRs, decreasing rates of HLA alloimmunization, and lowering risk of CMV transmission. It is generally considered equivalent to providing CMV-seronegative products for the prevention of transfusion-transmitted CMV [39]. In the United States, >95% of RBCs and platelets are leukocyte reduced. Plasma does not undergo leukocyte reduction by filtration, and the amount of whole blood leukocyte reduced is negligible at present. Other countries outside of the United States have adopted 100% leukocyte reduction.

(b) Irradiation

Irradiation of blood components is an established practice to prevent TA-GVHD. Blood components are exposed to a source of ionizing radiation that prevents the proliferation of viable T lymphocytes in blood components by creating DNA damage and blocking cell division. The dose of irradiation delivered should be 2500 cGy targeted to the central portion of the blood container and a minimum of 1500 cGy to any point at the periphery.

Irradiated cellular products are indicated for use in patients at risk of TA-GVHD, in particular patients with cellular immune deficiencies, recipients of marrow or peripheral blood progenitor cell transplantation, Hodgkin's disease patients, and fetal and neonatal recipients of intrauterine or exchange transfusions. Additionally, those at risk include recipients of cellular components from a blood relative and recipients of HLA-matched cellular components (see below). Hazards of irradiation include damage to the red blood cell membrane that can lead to potassium leakage and decreased posttransfusion survival. Irradiated components have a shelf life of no more than 28 days from date of irradiation but not to exceed the initial outdate of the product.

(c) Washing

Washing of RBCs and platelets can be performed to remove plasma proteins, electrolytes, or additives such as mannitol. The primary indication for washed components is to prevent severe allergic transfusion reactions. Washing can also be used to remove IgA-containing plasma for IgA-deficient recipients. Washing can result in significant cell loss. Washed components have a maximum shelf life of 24 h at 1–6 °C or 4 h at 20–24 °C. Only cellular components should be washed.

(d) Volume Reduction

Volume reduction involves centrifugation to remove a portion of the supernatant containing plasma and storage medium. It is indicated in patients who have circulatory dysfunction or low tolerance for extra volume, such as infants with cardiac dysfunction. It can be used as a measure to mitigate transfusion reactions, in particular transfusion-associated cardiac overload (TACO), as well as allergic reactions and ABO incompatibilities.

(e) CMV-Serology Testing

Reducing the risk of CMV can be achieved by testing for CMV or by leukoreduction. While CMV is a cell-associated virus, some free virus especially in recently infected persons means leukoreduction could leave a small remaining residual risk. CMV testing is by EIA to detect both CMV IgG and IgM. A general rule of "CMV safe" is a product with residual leukocyte count of $<1–5 \times 10^6$ white blood cells (WBCs) per unit. Specific patient populations including low birth weight neonates, fetuses requiring intrauterine transfusion, pregnant women, patients with primary immunodeficiencies, transplant recipients, and patients receiving chemotherapy or transplantation for malignant disease may be at risk of life-threatening CMV infection [40].

Alternatives to Transfusion/Mitigation Measures

Transfusion of human blood components carries significant risk of morbidity and mortality. Therefore, blood product administration should receive careful consideration, avoiding unnecessary transfusions. Increasing attention has been devoted in recent years to alternative measures to transfusion as risks of blood transfusions are further understood.

Many preoperative patients have undiagnosed anemia, at a rate estimated up to 75% [41]. Evidence, primarily been shown in orthopedic settings, has shown that treatment with preoperative erythropoietin and/or iron therapy reduces allogeneic blood transfusion associated with major surgical procedures [42, 43]. Further, perioperative transfusions can result in serious consequences such as lung injury, renal failure, and hemolysis. Therefore, identifying and treating anemia with intravenous iron and/or erythropoietin in the preoperative setting can provide substantial benefit in minimizing transfusions and potential harm. Various algorithms have been developed to guide the management of preoperative anemia [44, 45].

Patients on warfarin therapy often have significant coagulopathy due to inhibition of the vitamin K-dependent synthesis of coagulation Factors II, VII, IX, and X. While plasma transfusions are often prescribed for warfarin reversal, in many cases vitamin K treatment is sufficient. In the absence of bleeding, low doses of oral vitamin K are appropriate. If the INR is above 9, higher doses of vitamin K may be necessary. For rapid reversal of warfarin in a bleeding patient or for an urgent surgical procedure, transfusion of FFP is indicated. However, the use of 4-factor prothrombin complex concentrate (PCC) may provide faster and more efficacious therapy [46]. PCCs contain variable amounts of all the vitamin K-dependent factors and however pose a risk of thrombosis. A recent meta-analysis found that for urgent reversal of warfarin, vitamin K and coagulation factor replacement with PCCs resulted in a significant reduction in all-cause mortality, more rapid decrease in INR, and less volume overload compared to vitamin K plus coagulation factor replacement with FFP [47].

In bleeding patients, antifibrinolytics such as tranexamic acid (TXA) can be used to reduce bleeding and lessen the need for blood transfusions. TXA is a synthetic analog of lysine that inhibits plasminogen activation, reducing fibrinolysis of clots. TXA has widely demonstrated efficacy in reducing bleeding and blood transfusions [48, 49]. In addition to use in trauma, it has been used effectively as a modality to reduce perioperative anemia in a variety of obstetric [50, 51] cardiac surgery and orthopedic procedures [52–54].

Specific Populations

(a) Massive Transfusion

The historical definition of massive trauma is receipt of one or more blood volumes (≥10 units of RBCs) in 24 h or less. Massive blood loss is most often a sequela of traumatic injury and however can also occur due to ruptured aortic aneurysms, obstetric emergency, organ transplantation, and gastrointestinal bleeding, among others. Multiple additional complications can develop in patients requiring massive transfusion including hypovolemia, hypothermia, dilutional coagulopathy, tissue ischemia, shock, and electrolyte abnormalities.

Acute coagulopathy of trauma is a phenomenon that often occurs due to DIC, hypoperfusion leading to acidosis, and receipt of large volumes of cold blood products contributing to hypothermia and hemodilution. Hypoperfusion can further lead to systemic anticoagulation via increased activated protein C, increased fibrinolytic activity, and increased activity of thrombomodulin. Dilutional thrombocytopenia can occur if platelets are not replaced.

US hospital trauma centers accredited by the American College of Surgeons Committee on Trauma are required to have a massive transfusion protocol (MTP) [55]. MTPs specify who can activate the protocol and how, what products are issued, in what order, and at what ratios once the MTP is activated. Common approaches in trauma have been to administer components at high ratios of plasma and platelets to RBCs (1:1:1 or 1:1:2). The platelet component of the ratio refers to whole blood-derived platelets. An apheresis platelet can be considered clinically equivalent to 4–6 whole blood-derived platelets. There is evidence that early administration of a higher ratio of plasma and platelets to RBCs is beneficial in achieving hemostasis and reducing death due to exsanguination at early time points [56].

As bleeding becomes controlled, transitioning to goal directed (i.e., laboratory result based) rather than ratio directed occurs. Traditional laboratory tests to evaluate coagulation status such as PT/INR and aPTT may not be appropriate early in a massive transfusion setting as results may not be available in time. Alternatively, functional whole blood clotting measurements, such as thromboelastography (TEG) and rotational thromboelastometry (RoTEM), can guide transfusion therapy. Evidence is lacking however in published literature to support the accuracy of TEG and RoTEM, and there is concern for bias in the studies. Therefore it has been suggested that these tests remain only for research purposes, until better evidence becomes available [57].

(b) Obstetrics

In the obstetric population, postpartum hemorrhage (PPH) is a significant cause of morbidity and mortality. Pregnancy is associated with a hypercoagulable state, characterized by increases in coagulation factors (e.g., Factor VII, VIII), decreases in anticoagulant activity, and reduced fibrinolytic activity [58]. Also, coagulopathy is a component of many obstetric emergencies. If patients are seen in clinical consultation in advance, a routine type and screen can be obtained. However, in many cases, women present in active labor in which case blood products must be released emergently. Generally, women with PPH are treated with standard MTP; however some centers have MTP specific to pregnant women based on known physiological differences, such as relatively elevated fibrinogen levels in pregnant women. Use of products such as fibrinogen concentrate, TXA, and recombinant Factor VIIa (rVIIa) in obstetrics is controversial [29, 59]. Recent results from the WOMAN trial demonstrated that TXA reduces death due to bleeding in women with postpartum hemorrhage and that it should be administered as soon as possible following bleeding onset [50].

The transfusion medicine physicians and blood bank must have a fluid system of communication with the obstetricians, anesthesiologists, and operating room in order to prevent delay and consequent fetal and/or maternal morbidity and mortality.

Hemolytic disease of the fetus and newborn (HDFN) is a potentially lethal condition caused by maternal antibodies to red cell antigens on the fetus inherited from the father that are foreign to the mother. Maternal immunoglobulin G (IgG) antibodies are transported across the placenta and opsonize fetal red cells, leading to extravascular hemolysis. The most common cause of clinically significant HDFN is antibody to Rh(D). However as a result of effective use of Rh immune globulin (RhIG), the incidence of Rh HDFN has decreased from approximately 13–0.04% [60]. ABO antibodies are seldom a clinically cause of HDFN. Other blood group antigens such as Kell and Duffy can also cause maternal alloimmunization and HDFN. Obstetrics patients should be monitored with typing and antibody screening, and D-negative patients should be identified to receive RhIG.

(c) Neonates/Pediatrics

Transfusion practices in neonates differ from those in adults due to differences in physiology, response to transfusion, and etiology of blood loss. Common causes of transfusion in neonates include anemia of prematurity, hemolysis, iatrogenic blood loss, and thrombocytopenia. The transfusion of 10–15 mL/kg of RBCs over a 2–3 h period is expected to increase the hemoglobin by 2–3 g/dL, while a platelet transfusion of 10 mL/kg will typically increase the platelet count by 40,000–50,000/μL. As previously referenced, neonates should receive standard-issue RBCs as there is no evidence that fresher blood correlates with improved outcomes [61, 22]. Red cell exchange may be required in infants with severe hemolysis. The principal indication of exchange transfusion is to prevent potentially dangerous concentrations of bilirubin, usually indicated for total bilirubin levels >25 mg/dL. Other functions of exchange transfusion are to improve anemia and remove antibody. Neonates, particularly those who are premature who are CMV-seronegative, should receive leukocyte-reduced RBCs and platelet components due to increased risk of transfusion-transmitted CMV (TT-CMV) (see below).

Thrombocytopenia in neonates can result from congenital infections, congenital heart disease, sepsis, DIC, maternal immune thrombocytopenia (ITP), and neonatal alloimmune thrombocytopenia (NAIT). NAIT is analogous to HDFN, resulting from maternal alloimmunization against paternally inherited platelet antigens. Up to 80% of cases of NAIT in women of European ancestry are due to antibodies against human platelet antigen-1a (HPA-1a = GPII1a). Unlike HDFN, NAIT does not require previous sensitization and can occur in a first pregnancy. Treatment may begin in utero by giving intravenous immunoglobulin (IVIG) to the mother. After birth, the infant may require platelet transfusions and IVIG administration. Platelets should be antigen negative; however if antigen-negative platelets are not available, general blood bank inventory may yield an acceptable response. Incompatible plasma should be minimized by volume reduction or washing.

Transfusion in older infants and children is based on similar indications as in adults. Clinical decisions for blood transfusions in children are affected by differences in blood volume, circulatory capacity, and normal hemoglobin and hematocrit ranges for the specific age group as well as other relevant laboratory parameters. As mentioned above, pediatric patients should have a hemoglobin transfusion threshold of 7 g/dL and receive standard-issue RBCs [22].

(d) Immunized Patients

Patients who receive chronic or multiple transfusions, such as those with hemoglobinopathies (e.g., sickle cell disease), are at risk of developing alloimmunization to red cells and subsequent hemolytic reactions [62]. Antigen-matched RBCs are of benefit in such patients. In particular, matching of Rh and Kell antigens is critical. Extended red cell typing (i.e., Rh, K, Jk, Fy, Ss) before initial transfusion enables facilities to provide partially matched RBCs for transfusion. Molecular methods are increasingly employed for genotyping. The practice of determining blood group genotype to predict phenotype is termed "molecular immunohematology." Genotyping is not consistently predictive of phenotype however due to mutations such as silencing.

Immunization to platelets is due to HLA or platelet-specific antibodies and can result in failure to achieve an expected platelet count increment following transfusion, termed platelet refractoriness (see above).

Adverse Events and Side Effects of Transfusion

(a) Immediate (Table 36.3)

(i) Acute hemolytic transfusion reactions (AHTRs) are most often the results of intravascular hemolysis of donor red blood cells caused by preformed alloantibodies in the recipient's circulation. AHTRs also result less commonly from transfusion of plasma containing antibodies against the recipient's red cell antigens. Fatal AHTRs most commonly result from the transfusion of ABO-incompatible blood. The incidence of AHTRs ranges from 2.5 to 7.9 per 100,000 units transfused [63], and mortality is <5% when recognized and treated promptly.

Table 36.3 Immediate adverse events/side effects of transfusion

Adverse event/side effect	Incidence per unit transfused
Acute hemolytic transfusion reaction (AHTR)	2.5–7.9 per 100.000
Fatal hemolytic reaction	1:972,000
Allergic reaction	1.12–3.3 per 100
Anaphylactic reaction	8 per 100,000
Febrile, nonhemolytic transfusion reaction (FNHTR)	
Non-leukocyte reduced	1–2.2 per 100
Leukocyte reduced	<0.2 per 100
Transfusion-related acute lung injury (TRALI)	1 per 5000
Transfusion-associated cardiac overload	0.1–1 per 100

Signs and symptoms of an AHTR can develop following as few as 10–15 mL of incompatible blood. The most common presenting symptom is fever with or without chills and rigors. Reactions may be accompanied by abdominal, chest, flank, or back pain. Severe reactions may progress to hypotension, dyspnea, shock, and DIC. Organ dysfunction notably renal failure can be a later complication.

The pathophysiology of AHTRs involves the binding of antigen-antibody complexes in the recipient, which activates the complement cascade leading to intravascular hemolysis. Laboratory findings of AHTRs demonstrate markers of hemolysis including decreased hematocrit, hemoglobinemia, hemoglobinuria, elevated lactate dehydrogenase, hyperbilirubinemia, and low haptoglobin.

The most important step in treatment is prompt recognition and immediate cessation of the blood transfusion. Saline should be infused to maintain venous access, treat hypotension, and maintain renal blood flow. Mechanisms to prevent/treat renal dysfunction should also be implemented. Other steps in treatment should be guided by the clinical condition of the patient.

(ii) Allergic transfusion reactions (ATRs) range in severity from very mild to life-threatening. A mild allergic reaction consists of the outbreak of urticaria (hives) and/or itching and/or erythema in response to a transfused allergen. ATRs often develop very soon, often within minutes of the start of a transfusion. While incidence rates have been reported as low as 112.2/100,000 transfusions, other studies have cited incidences ranging from 1/100 to 1/33 transfusions. It is likely that many mild allergic reactions are either not recognized as related to transfusion or not reported. Incidence rates along the higher end of the reported spectrum conform more closely to clinical practice [63–66].

Mild ATRs manifest as raised, erythematous and swollen cutaneous areas accompanied by symptoms of pruritus, burning and/or stinging. The rash typically lasts hours to days but can respond very quickly to antihistamines. More severe reactions involve angioedema, swelling, and fluid accumulation beneath the skin. Angioedema can affect the throat and airway passages, potentially leading to severe respiratory distress.

ATRs are considered to be Type I hypersensitivity reactions caused by preformed IgE antibody in the recipient reacting to allergens transfused in the component. However it is recognized that other pathophysiologic mechanisms might exist that have not yet been elucidated [67]. Antibodies against IgA in persons with IgA deficiency can be associated with severe reactions. Other known triggers include patient antibodies to haptoglobin, penicillin, and the C4 determinant of complement.

Initial treatment involves discontinuing the transfusion while maintaining intravenous access. Mild ATRs may not need treatment or will often respond to antihistamines such as diphenhydramine. Patients with a history of allergic reactions should be pretreated with antihistamines; however other patients should not be empirically treated [67, 68]. If antihistamines are not sufficient, treatment with oral prednisone or steroids may be beneficial. In mild allergic reactions with no airway involvement, the transfusion often can be restarted safely. If symptoms recur or worsen, the transfusion will need to be discontinued. More severe reactions involving the respiratory passages may require intubation.

Severe allergic (anaphylactic) transfusion reactions may be fatal. The incidence of anaphylactic reactions has been reported at 8/100,000 [63]. Anaphylactic reactions typically include symptoms of urticaria and angioedema but can also involve severe hypotension, shock, and loss of consciousness along with dyspnea, wheezing, and stridor. Patients may require intubation. Cardiac manifestations may include tachycardia, arrhythmia, shock, and cardiac arrest. Additional symptoms such as vomiting, diarrhea, nausea, and cramping may accompany the reaction. If an anaphylactic reaction occurs, the transfusion should be stopped immediately. The patient should be provided supportive care, including oxygen to maintain oxygenation and intravenous fluids and epinephrine to stabilize hypotension. Patients receiving epinephrine should receive cardiac monitoring due to its arrhythmic potential.

(iii) Febrile nonhemolytic transfusion reactions (FNHTRs) are defined as an increase in temperature ≥1°C and/or chills, rigors, and discomfort occurring during or within 4 h of a transfusion. Clinical determination of an FNHTR is a diagnosis of exclusion. Chills and rigors in the absence of a fever may constitute a FNHTR if they represent a symptom complex consistent with a febrile reaction. Premedication with antipyretics such as acetaminophen explains why some FNHTRs may present without a rise in temperature. Symptomatic treatment with antipyretics is appropriate; however patients should not be treated prophylactically as the first sign of a serious intravascular hemolytic reaction can be a temperature elevation [69].

The incidence of FNHTRs in a normal population is approximately 1 out of 100 non-leukocyte-reduced RBC transfusions and approximately 2.2 out of 100 non-leukocyte-reduced platelet transfusions. If the components are leukocyte reduced, the rate of reactions in both RBCs and platelets falls to <0.2 out of 100 transfusions. In the hematology-oncology population, the rate of FNHTRs with non-leukocyte-reduced RBCs is approximately 6.8 per 100 transfusions, and for non-leukocyte-reduced platelets, the rate of FNHTRs is over 30%. If the components are leukocyte reduced, these rates fall to 0.3 out of 100 RBC transfusions and fewer than 2 per every 100 platelets transfusions in the hematology-oncology population [70].

There are thought to be two pathophysiologic mechanisms responsible for FNHTRs. The first mechanism implicates the infusion of soluble biologic response modifiers, thought to be responsible for most FNHTRs to platelets. Under this paradigm, cytokines (IL-1, IL-6, TNF-α) accumulate during storage, leading to the production of prostaglandin E2, which acts on the hypothalamus to induce pyrogenic symptoms. The second mechanism is antibody-mediated, thought to be responsible for most FNHTRs to RBCs. In this mechanism, recipient HLA or human neutrophil antigen (HNA) antibodies react with donor WBCs to elicit an antibody-antigen reaction leading to the release of fever-inducing inflammatory molecules. The latter type of reaction is more likely to occur in previously transfused patients or multiparous women.

(iv) Transfusion-related acute lung injury (TRALI) is the sudden onset of respiratory distress and pulmonary edema. Commonly cited Canadian Consensus Conference criteria define TRALI as acute lung injury occurring during or within 6 h of transfusion with hypoxemia and PaO2/FiO2 ≤300 or SpO2 ≤90% on room air, bilateral infiltrates on chest X-ray, no evidence of left atrial hypertension, no preexisting acute lung injury, and no temporal relationship to an alternative risk factor for acute lung injury [71]. Reporting and recognition of TRALI are highly variable, making estimates of reaction rates difficult. The incidence of TRALI is estimated at approximately 1 in 5000 transfusions [72] although owing to variable reporting and clinical recognition a vast range of incident rates have been cited ranging from 1:432 WBD platelets to 1:557,000 RBCs [73]. The fatality rate is 5–10%.

Signs and symptoms include dyspnea, fever, hypoxia, pulmonary edema, hypotension, normal central venous pressure, and normal pulmonary wedge pressure. TRALI is distinguished from TACO by the lack of cardiac involvement including lack of circulatory overload, normal B-type natriuretic peptide (BNP) levels, and lack of response to diuresis. TRALI is secondary to non-cardiogenic pulmonary edema. Laboratory tests in the acute setting are of limited value as they are not diagnostic of TRALI.

TRALI results from neutrophil and pulmonary endothelial activation. The majority of cases (~75%) are associated with HLA (predominately Class II) and HNA antibodies in the donor reacting with the recipient's WBCs. The result is leukoagglutination, activation of the complement cascade, and cytokine release, leading to fluid accumulation in the alveoli. By excluding plasma collected from female donors, TRALI incidence declined. Less frequently (~5%), TRALI occurs due to recipient antibodies reacting against donor leukocytes. The remaining ~20% of cases are thought to be due to a nonimmune mechanism, whereby bioactive lipids and CD4 ligand in the product prime the patient's neutrophils that are sequestered in the pulmonary vasculature and a second stimulation, such as infection or tissue injury results in the release of vasoactive mediators. Both antibody-mediated and non-antibody-mediated TRALI are thought to represent common pathways of neutrophil priming, pulmonary vasculature activation, endothelial injury, and capillary leak.

If TRALI is suspected, the transfusion should be immediately discontinued. Medical management is supportive and should entail blood pressure and respiratory support, in some cases requiring intubation. Diuresis is not indicated. Patients generally recover in 2–4 days with supportive care. Patients typically do not have recurrent episodes with further transfusion as TRALI is generated as a result of a specific patient reaction to a particular character-

istic of the donated component. Indications for subsequent transfusions should not be adjusted based on the occurrence of a TRALI reaction.

(v) Transfusion-associated circulatory overload (TACO) represents a constellation of symptoms including respiratory distress, pulmonary edema, and evidence of fluid overload temporally related to the administration of blood components. Susceptible populations include the very young, the elderly, and patients with small total blood volume or impaired cardiorespiratory status. It is frequently unrecognized and is a leading cause of transfusion-associated fatalities reported by the US FDA. Signs and symptoms of TACO include dyspnea, hypertension, cough, chest tightness, pulmonary edema, orthopnea, jugular venous distension, and cardiac arrhythmias. The incidence of TACO has been reported ranging from 1/100 to 1/1000 transfusions [74]. The wide variability in reported incidence reflects under-recognition and underreporting of the condition.

The diagnosis of TACO is made based on cardiogenic pulmonary edema. BNP levels, which are elevated in TACO, can be helpful to distinguish TACO from TRALI or other etiologies. Treatment includes diuresis, oxygen supplementation, and sitting the patient upright. Transfusions in a patient at risk of TACO should be administered slowly and cautiously. The rate of transfusion in a normal setting should be 2 mL/kg/h; in a patient at risk of TACO, blood components should be administered more slowly.

(b) Delayed (Table 36.4)

(i) Delayed hemolytic transfusion reactions (DHTRs) are characterized by red cell destruction in the recipient's circulation due to an immune response to a foreign red cell antigen. DHTRs are most commonly an unexpected anamnestic response in a patient previously exposed to a foreign antigen either through pregnancy or transfusion, reflecting the evanescent nature of many alloantibodies. Despite a negative antibody screen, patients with DHTRs have developed an antibody that remains at low undetectable levels. Re-exposure to the antigen upon transfusion produces an anamnestic antibody response resulting in increased production of IgG antibodies that are capable of reacting with any transfused cells present. On rare occasions DHTRs result from primary alloimmunization to a red cell antigen.

DHTRs are characterized by an unexpected decrease in hemoglobin or failure to achieve the expected increase in hemoglobin posttransfusion. Clinical signs of hemolysis usually appear 3–10 days posttransfusion. Other signs and symptoms include fever, chills, jaundice, malaise, back pain, and rarely renal failure. Patients may be asymptomatic, in which case the reaction is detected only upon recognition of a more rapid decline than usual in the patient's hemoglobin or lack of expected rise in hemoglobin.

DHTRs are usually extravascular, and the involved antibodies rarely fix complement. Consequently, they are often less severe than AHTRs. DHTRs are often associated with Rh and Kell system antigens. The DAT will be positive in most cases due to coating of the transfused donor red cells with recipient antibody. Anemia and indirect hyperbilirubinemia may result. Other laboratory findings may include an increase in LDH, a decrease in haptoglobin, and reticulocytosis. To prevent future reactions, the patient should receive antigen-negative RBCs for any necessary transfusions.

Delayed serologic transfusion reactions are defined as the presence of a newly identified alloantibody in the absence of evidence of increased red cell destruction. The incidence of delayed transfusion reactions is approximately 1 in 2500 RBC transfusions; serologic reactions occur twice to five times as frequently as hemolytic reactions [75].

(ii) Transfusion-associated graft-versus-host disease (TA-GVHD) is a nearly 100% fatal syndrome caused by the infusion of immunocompetent donor lymphocytes which attack the tissue and organs of an immunocompromised recipient. The incidence of TA-GVHD was reported as approximately 1:930,000 components transfused in the United Sates in 2011 [76]. The incidence increases in countries with more homogenous populations such as Japan, where the risk of developing TA-GVHD has been estimated at 1/874.

Upon transfusion, viable lymphocytes in the product engraft into the recipient and mount an insuperable immune response in an immunocom-

Table 36.4 Delayed adverse events/side effects of transfusion

Adverse event/side effect	Incidence per unit transfused
Delayed hemolytic transfusion reaction (DHTR)	1 per 2500
Transfusion-associated graft-versus-host disease (TA-GvHD)	1 per 930,000
Posttransfusion purpura	Unknown
Iron overload	> 10–20 red cell units
Transfusion-related immunomodulation (TRIM)	Unknown

promised host. Donor T cells proliferate and recognize the host as foreign, while the recipient's immune system fails to recognize the donor cells as foreign. The bone marrow in TA-GVHD is of recipient origin and therefore a targeted organ. The attack against host bone marrow often results in bone marrow failure in addition to gastrointestinal tract, liver, and skin involvement, causing most cases to be fatal. TA-GVHD can also occur in immunocompetent hosts, in which case the recipient is typically heterozygous for HLA antigens, while the donor is homozygous for an HLA haplotype in the recipient. The donor mounts an immune response against unrecognized HLA antigens in the host, while evading HLA-mediated immune detection by the host. Reactions in immunocompetent patients often occur in homogenous populations [77].

Patients at risk of TA-GVHD are those with cellular immunodeficiencies, including congenital cellular immunodeficiencies (e.g., DiGeorge syndrome), hematopoietic progenitor stem cell recipients, acute leukemia and other hematologic malignancies, patients undergoing treatment with purine analog drugs (e.g., fludarabine, cladribine, deoxycoformycin), Hodgkin lymphoma, and low birth weight infants or recipients of intrauterine transfusion. Immunocompetent patients at risk include those receiving blood from genetic relatives and HLA-matched products. Patient with HIV infection and organ transplants are not at increased risk. Most patients on immunosuppressive regimens for solid tumors are also not at risk.

TA-GVHD is characterized by clinical signs and symptoms of rash, fever, hepatitis, diarrhea, and hypoproliferative pancytopenia. TA-GVHD has a 90–100% fatality rate due to pancytopenia that renders patients susceptible to overwhelming infection and multiorgan failure, resulting in death. Common treatment approaches include the administration of immunosuppressive or immunomodulatory medications and occasionally stem cell transplant. All treatment modalities to date have yielded limited success, resulting in a focus on prevention.

Irradiation is effective in preventing TA-GVHD by causing donor lymphocytes to become incapable of replicating. In order to prevent TA-GVHD, cellular blood products are irradiated with a minimum dose of 15 Gy to any point of the container and 25 Gy at the center of the container [78]. Countries with homogenous populations such as Japan have chosen to irradiate all cellular blood components. Although irradiation has a high success rate in preventing TA-GVHD, there have been at least five reported cases of TA-GVHD in patients who received irradiated products. In some of these cases, however, the recommended dose of irradiation was not delivered [77]. Irradiation remains a generally proven method of prevention of TA-GVHD. Leukoreduction, in contrast, reduces risk but is not sufficient to prevent TA-GVHD due to residual active T lymphocytes remaining in the component following treatment. However data from the UK's hemovigilance system, Serious Hazards of Transfusion (SHOT), demonstrate a significant reduction in cases of TA-GVHD following the implementation of leukocyte reduction, lending support to some benefit [79]. Pathogen reduction appears effective in preventing TA-GVHD.

(iii) Posttransfusion purpura (PTP) is an immune thrombocytopenia resulting from antiplatelet antibodies, most often directed against HPA-1a. PTP is a rare condition; therefore estimates of incidence are difficult to establish.

Patients present with purpura and thrombocytopenia on average 5–10 days following a transfusion, although presentation can range from 1 to 24 days. The typical patient is a middle-aged or elderly woman with a recent history of transfusion, with a 5:1 female to male ratio. The platelet count usually decreases to <10,000/μL. Widespread purpura is accompanied by findings including epistaxis, bleeding from mucous membranes, bleeding of the gastrointestinal and urinary tracts, and in severe cases intracranial hemorrhage and death. The mortality rate is approximately 10%, resulting primarily from intracranial hemorrhage.

The transfusion precipitating PTP causes an anamnestic immune response against specific platelet antigens in a patient with previous exposure to platelet antigens through pregnancy or transfusion. While the platelet-specific alloantibodies are most often directed against HPA-1a (~70% of cases), other HPA antigens have been implicated. Platelet antibody screening and identification can be of diagnostic utility. The antibody has pan-reactive activity, destroying autologous platelets as well as transfused antigen-negative platelets. Understanding of the pathogenesis behind the destruction of autologous platelets and antigen-negative platelets is incomplete.

First-line treatment in PTP consists of IVIG therapy. Appropriate IVIG treatment can increase the platelet count to >100,000/μL in 4–5 days; however improvement can be seen within hours. The duration of thrombocytopenia in untreated patients

is approximately 2 weeks. Plasma exchange and steroid therapy are indicated as second-line treatment modalities. Leukoreduction may have some efficacy in reducing the incidence of PTP, although cases of PTP have been precipitated by the infusion of leukocyte-reduced components or whole blood [80]. For future platelet transfusions, patients should be given platelet-specific antigen-negative platelets, which usually will be HPA-1a-negative.

(iv) Iron overload can result as a complication of chronic red cell transfusion therapy. Patients with conditions such as congenital anemia (e.g., sickle cell disease and thalassemia) requiring chronic red cell transfusions are at particular risk.

One mL of red blood cells contains 1 mg of iron, and the iron content in each red cell transfusion is approximately 200–250 mg. The normal rate of excretion of iron is 1 mg/day. There is no physiologic mechanism for excreting excess iron. Thus, in patients requiring chronic transfusions, iron buildup can cause organ damage, in particular of the heart, liver, spleen, and endocrine system. Symptomatic iron overload occurs when body iron reaches 10–25 g, the equivalent of 50–100 red cell transfusions. Clinical signs and symptoms of iron overload include hepatomegaly, cirrhosis, congestive heart failure, cardiomyopathy, and endocrine dysfunction. Hypothyroidism and diabetes may occur.

Iron-chelating agents are beneficial as treatment in patients receiving chronic red cell transfusions. Iron chelators bind to iron in the body and aid in its elimination through urine and feces. Iron chelation should be considered in any patient anticipated to require chronic red cell transfusion therapy that has already received greater than ten transfusions. Other indicated treatment options are exchange transfusion and therapeutic phlebotomy.

(v) Transfusion-related immunomodulation (TRIM) relates to the effects of blood transfusion on the immune system. While the most evident immunological effects of transfusion are red blood cell and HLA alloimmunization, blood products have additional and profound effects on recipient immunity. The postulated mechanism is immunomodulation via enhanced humoral immunity in combination with depressed cellular immunity [81]. Accepted effects of TRIM include improved allograft survival following transfusion prior to organ transplantation and reduced likelihood of recurrent spontaneous abortion following transfusion of allogeneic blood. Controversial effects of TRIM include a potential increase in cancer recurrence or metastasis, increased postoperative infection, and improvement in certain autoimmune conditions, such as Crohn's disease. However, data are conflicting in studies regarding the impact of transfusion on cancer, infection, and autoimmunity, and no effect has been conclusively shown [75, 82–84].

Transfusion-Transmitted Infection (Table 36.5)

Any virus, bacterium, parasite, and other pathogens that can be transmitted to a recipient from blood or blood components prepared from donations are classified as a transfusion-transmitted infection (TTI). A high level of hemovigilance with respect to emerging or potential TTI has been in place since the transmission of HIV and HCV infections to thousands of blood product recipients in the 1980s and early 1990s. Surveillance for emerging pathogens is ongoing, and detailed evaluations of the risks of specific pathogens have been developed and with the knowledge base updated regularly as new information becomes available [85]. Although TTI have contributed a relatively small proportion to the total burden of infection from all routes, each specific infection is acquired, and the prevention of TTIs remains paramount. Monitoring the rates of infection in donors and residual risk of known TTI agents as well as for the emergence of new agents (Zika and Ebola), reemergence of agents (yellow fever, malaria) continues to be one of the primary tools used

Table 36.5 Transfusion-transmitted infections

Agent	Incidence per unit transfused
Human immunodeficiency virus (HIV)	1 per 1500,000
Hepatitis C (HCV)	1 per 1,200,000
Hepatitis B virus (HBV)	1 per 765,000
Hepatitis A virus (HAV)	Infrequent
Hepatitis E virus	None reported in United States[a]
Syphilis	No recent cases
Bacterial contamination	
Apheresis platelets	1 per 1500–1 per 2747
Red blood cells	1 per 1000,000
Babesia	> 256 cases reported
Malaria	1 case q 2 years
T. cruzi	Infrequent, platelet transfusion association in United States
CJD	None reported
vCJD	Five cases reported
West Nile virus	~30 cases, 7 since testing began in 2003
Human T-lymphotropic virus	1 per 2,400,000
Zika virus	No cases reported to date in the United States[b]

[a]Multiple and increasing cases documented throughout Europe and Japan since 2004

[b]Documented platelet transfusion-transmission in Brazil

to define donor eligibility policies focused on reducing the risk of transfusion-transmission.

Donors with reactive infectious disease screening test results may have presented to donate without knowledge or understanding they were infected or may donate because they did not disclose a deferrable risk on the donor health history questionnaire. Confirmatory testing ascertains infected donors who are then notified of the infection and counseled to seek medical care. Thereafter, donors may be asked to participate in local blood center efforts to assess which behaviors may be associated with acquisition of the infection. Larger-scale hemovigilance efforts are also common and serve the purpose of monitoring for changes in the risk behaviors disclosed by donors.

Blood donor screening and blood testing approaches for preventing the risk of each pathogen vary according to the agent, available screening technologies, the performance characteristics of those technologies (i.e., sensitivity and specificity), and the costs associated with screening. The first approach to preventing TTI, donor screening, involves asking donors during the eligibility assessment about behaviors associated with infections, to defer those who report risky behaviors associated with specific infections. Deferral based on donor health history reduces the risk of infections such as malaria and for persons with history of infections such as hepatitis or babesiosis. Blood testing via serological screening for antibodies to pathogens or direct detection of antigens has been the mainstay of donation screening for decades. The development of amplification assays which measure viral nucleic acids led to the adoption of the minipool (MP) nucleic acid test (NAT) screening for HIV, HCV in 2000, and HBV in 2009 in the United States. The pools consist of 6–16 donations. If the MP is reactive, the individual donations (ID) are tested separately to identify or "resolve" which donor is positive. Many countries have adopted ID NAT screening because of the higher sensitivity to detect low viral load infections in donations.

The use of pathogen reduction technologies may alter the focus of blood safety in the future (see below), but at this time screening donors and donations to prevent the risk of TTI is the primary approach to ensuring recipient safety.

(a) Viral

Viruses

As described previously, donated blood is screened for a range of infections that are transfusion-transmissible. The reporting of AIDS-like illnesses in hemophiliacs and blood recipients starting in late 1982 and early 1983 lead to the documentation of transmission in pooled plasma products and labile blood components [86–89]. As available screening advanced from serology assays to the combined use of serology and nucleic acid testing, the residual risk of HIV, HCV, and HBV has decreased. The current risk of transfusion-transmitted HIV in the United States is estimated to be 1:1500,000 donations and for HCV 1 in 1,200,000 donations [90]. Hepatitis B virus continues to have a slightly higher risk owning to the dynamics of infection by the pathogen [91]. The current estimated residual risk is 1:765000 [92].

The US blood supply is also screened for HTLV, WNV, and ZIKV. The approach to screening is highly variable. Serological assays are used for HTLV. For WNV, MP NAT is conducted unless local, seasonal outbreak activity is identified in the region of the blood center. When this occurs, screening is switched to ID NAT. In 2016, the United States began screening all donations for ZIKV using ID NAT. Other jurisdictions have adopted travel-related deferrals instead of donation screening for ZIKV.

(b) Bacteria

(i) Spirochetes – Syphilis (*Treponema pallidum*) screening has been conducted since the 1940s. Today the risk of TT syphilis is very small because of the refrigerated storage conditions of red cells [93] with the last reported TT syphilis case in the United States occurring in 1966 [94]. The standard approach to donation screening for syphilis is to use an EIA with confirmation by using a *T. pallidum* hemagglutination assay (TPHA). Rapid plasma reagin (RPR) assays can be used to determine if the infection was recent. When the infection is transmitted to a recipient by transfusion, signs and symptoms appear a few weeks later; these can include macular lesions on the palms, headache, arthralgia, fever, peripheral lymphadenopathy, and, rarely, jaundice [95].

(ii) Bacterial contamination of platelets remains one of the most important infectious threats in transfusion. Every year in the United States, the FDA receives two to five case reports of fatalities resulting from bacterial contamination of platelets [96]. Passive reporting of septic transfusion reactions has been documented to significantly miss the majority of these events [97]. *Staphylococcus* spp. are a common pathogen causing bacteremia and sepsis in patients. However, the list of bacteria that have been transmitted by transfusion is wide ranging. In the last 5 reporting years for which data are available, fatal TT bacterial infections have been caused by all of the following: *Staphylococcus aureus*, *Escherichia coli*, *Staphylococcus epidermidis*, *Morganella morganii*, *Streptococcus viridans*, *Streptococcus pneumoniae*, *Staphylococcus warneri*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Pseudomonas fluorescens*, and *Acinetobacter* sp. [96].

The Code of Federal Regulations (21 CFR 606.145(a)) states: "Blood collection establishments and transfusion services must assure that the risk of bacterial contamination of platelets is adequately controlled using FDA approved or cleared devices or other adequate and appropriate methods found acceptable for this purpose by FDA." Partial prevention of TT bacterial infection resulting from bacterially contaminated platelet transfusion is achieved through bacterial culture shortly after collection. However, the sensitivity of this process has been demonstrated to be only between 11 and 47% [98]. Additionally, rapid testing (within 24 h of transfusion of apheresis platelets or within 4 h of transfusion for whole blood-derived platelets) or pathogen reduction has been recommended in an FDA draft guidance [98].

(c) Parasites

While there are many potential parasites of concern, in the US context, three parasites are of greatest concern. Each is transmitted by a different vector and addressed by a different donor or donation level strategy.

(i) Babesia

Babesia spp. are transmitted to humans by ticks in the genus *Ixodes*. Babesia is endemic to parts of the United States and is the most common specific infection leading to fatalities in red cell transfusion recipients [96]. *Babesia microti* is present in New England and the upper Midwest (Wisconsin and Michigan) [99]. Over the last 20 years, hundreds of cases of TT babesiosis have been documented in the United States [100, 101]. In addition to *B. microti*, other species of Babesia have been transfusion transmitted. In areas of the country with the highest risk, blood centers may screen donations for *B. microti* using either an antibody test or combined NAT and antibody test. Parasitemic donations pose the highest risk to recipients, and so screening for the parasite genome by. NAT is the best method to reduce risk [99].

(ii) Malaria

Malaria is primarily transmitted to humans by female mosquitoes of the genus *Anopheles*. The risk of transfusion-transmitted *Plasmodium* spp. (malaria) is mitigated, but not eliminated through the use of temporary or permanent deferral of donors who travel to visit or emigrate from malaria endemic areas. The CDC monitors and updates locations with endemic malaria. This effort is used as a primary resource to determine if a donor traveled to a malaria endemic area. However, cases of TT malaria continue to occur and are often the result of temporarily deferred, semi-immune donors who have no signs or symptoms of infection and present to donate after travel deferral period has lapsed. On average, only one case of transfusion-transmitted malaria occurs in the United States every 2 years [102].

(iii) *Trypanosoma cruzi* (*T. cruzi*)

T. cruzi is the etiologic agent for Chagas' disease. *T. cruzi* is endemic to South, Central, and southern portions of North America and is transmitted to humans through the blood meal of triatomine beetles (known as the kissing bug because the common location to take a blood meal is from human lips). Universal screening for antibodies to *T. cruzi* in donated blood in the United States was adopted in 2007. In 2010, the policy was modified to allow one-time screening of donors because of lack of evidence of substantial incident infection in US blood donors.

(d) Prions

Transmissible spongiform encephalopathies (TSEs) are an unresolved concern and risk to blood transfusion recipients, albeit the risk of TT TSE is very small at this time [103]. The infectious agents are unique because they are misfolded proteins that are capable of being replicated by cellular machinery but do not themselves have DNA or RNA. The clinical course of infection can lead to the development of neurological disease in a short period of time but is more common as an insidious infection that may not lead to clinically apparent disease for up to 20 years. TSEs of highest concern to blood safety are Creutzfeldt-Jakob disease (CJD), variant Creutzfeldt-Jakob disease (vCJD) also known as mad cow disease, and chronic wasting disease (CWD), but there are several other known prion diseases that could be TT [104]. TSEs are primarily transmitted by consumption of contaminated neuronal tissues or through direct exposure to blood of an infected animal or human. The largest vCJD outbreak occurred in the 1980s and early 1990s from the human consumption of contaminated cattle in the United Kingdom and led to the documentation of at least five transfusion-transmitted infections [105]. No cases of transfusion-transmitted CJD have been identified, despite recent extensive lookback studies [106]. Human genetics play significant role in disease progression. Substantial effort to develop screening tests for TSEs with a focus on vCJD or to develop blood filters that can remove the misfolded proteins has been undertaken, but none are approved in the United States.

New Safety Considerations

(a) Pathogen reduction

There are many forms of pathogen reduction technology available internationally, and two specific pathogen reduction technologies have been approved by the FDA for use in the United States. A solvent detergent-treated pooled human plasma blood product (Octaplas™)

reduces the risk of TTI for patients requiring replacement of clotting proteins in certain medical conditions, such as bleeding disorders. An amotosalen and UV light process (Intercept™) for plasma is intended to be used for the ex vivo preparation of pathogen-reduced, whole blood-derived, or apheresis plasma in order to reduce the risk of TTIs. When Intercept™ is used for platelets, the ex vivo process involves apheresis platelet components suspended in platelet additive solution or plasma to reduce the risk of TTIs, including sepsis, and to potentially reduce the risk of TA-GVHD. Posttransfusion platelet recoveries in patients are significantly reduced following Intercept treatment; however evidence indicates that the effect on hemostasis is at most mild [107, 108, 109, 110, 111, 112, 113].

Outside of the United States, technologies with Council of Europe (CE) mark are available and in use. Solvent-detergent treatment (Octoplas™), methylene blue with visible light (Macopharma and Springer), amotosalen and UV light (Intercept™), and riboflavin with UV light (Mirasol™) are available for fresh frozen plasma [114]. For platelets, amotosalen and UV light (Intercept™) and riboflavin with UV light (Mirasol™) also are available. Currently no red cell technologies are approved, but research, including clinical trials, is being conducted for red cell-specific inactivation (Intercept™) and whole blood inactivation (Mirasol™). Previously, some red cell inactivation technologies were developed but were discontinued due to evidence of adverse consequences focused on the formation of red cell antibodies. Adoption of pathogen reduction technologies has been limited due to fiscal constraints on blood centers and hospitals.

(b) Bacterial testing of platelets
Bacterial culture of platelets shortly after collection is the primary screen used to identify evidence of contamination in platelets. However, as noted above, the sensitivity of these devices is low. Point-of-release testing devices for bacteria using rapid testing were cleared in the United States. These devices are designed to detect antigens common to all gram-positive and gram-negative bacteria. One such device (the Platelet PGD Test) is cleared by the FDA as a "safety measure" and can be used to extend platelet storage from 5 to 7 days.

Patient Hemovigilance

Hemovigilance or biovigilance, referring to a set of transfusion-specific surveillance procedures, is the vein to vein from donor to recipient program to monitor, assess, and respond to identified risks associated with transfusion [115]. The risks are not restricted to infectious threats, and monitoring for noninfectious threats is arguably more important because the occurrence of noninfectious adverse events is more frequent. Many countries have formal passive or active hemovigilance systems. Most systems are passive because of the very high cost of active monitoring of transfusion recipients. Semi-active systems include those found in many European countries where if an adverse event is identified it is tracked and recorded, but without evidence of an adverse transfusion event, recipients are not specifically monitored. In addition, the Serious Hazards of Transfusion (SHOT) program in the United Kingdom is a systems used to report adverse events, relying on voluntary reporting [116]. Both AABB and US CDC have developed and promoted the adoption of hemovigilance. The United States has a small-scale hemovigilance system based on the voluntary participation by hospitals. Only in the State of Massachusetts are all hospitals required to report adverse events to the US system.

Risk-Based Decision-Making

Risk-based decision-making (RBDM) is an initiative that has seen increasing adoption by blood collection organizations [117, 118]. The genesis of the effort was the recognition of competing priorities in decision-making for blood safety and the desire to establish a framework that could permit more consistent application of tools and methods to address the range of blood safety decisions blood-collecting organizations face. These efforts are partially borne out of insufficient resources to address all safety threats coupled with the recognition that zero risk is an unattainable objective. An initiative developed by several large blood operators has established a structured approach for defining and assessing risk (Fig. 36.1).

The structured approach relies on the development of evidence from a set of evaluations. The evaluations include options assessment, risk tolerability, risk assessment, health economics, and stakeholder engagement. Together these evaluations help blood operators (and regulators) understand the implications of different mitigation strategies. The RDBM framework is seeing increasing adoption in countries including Canada, Australia, and the United Kingdom.

Health Economics and Cost-Effectiveness

Linked to RBDM, but also relevant from the standpoint of efficiency in healthcare, cost-effectiveness analysis (CEA) and other forms of health economic assessments are a set of methods used to assess the trade of money spent and gain in health achieved. Several review publications are available which provide in-depth discussion of the methods and

Fig. 36.1 Structured approach for defining and assessing risk

limitations of these analyses [119–123]. Overall, the use of cost-effectiveness in blood safety and transfusion medicine intervention decision-making has been limited. The recognition of the potential utility of these methods is evident, but in few countries or settings are blood collectors expected to operate under a fixed budget, requiring formal evaluation be conducted of the benefits achieved for monetary resources expended of each blood safety and transfusion intervention. Continued pressure to reduce or lower costs on hospitals, clinicians, and blood providers increases the relevance of CEA, but may not lead to formal use of these methods until budgets are recognized as finite. In addition, there are multiple competing choices that have to be made at the same time.

Many of the blood safety decisions or newly developed interventions do not conform to traditional thresholds considered cost-effective. In particular, infectious disease screening, including NAT for HIV, HBV, and HCV, and HTLV and *T. cruzi* serology testing have been shown to have cost-effectiveness results near or above $1000,000 or more per quality-adjusted life year (QALY) gained. These results are in contrast to a common rule of thumb used in other sectors of health and medicine where $100,000 per QALY gained is considered a threshold that can be used to define an intervention as cost-effective. The cost-effectiveness of adopted blood safety interventions across different countries rarely meets this threshold or equivalent thresholds in other currencies. Most countries appear willing to dedicate healthcare resources to blood supply safety in excess of that for other sectors of healthcare, implying the societal willingness to pay for blood safety exceeds that of other areas of healthcare. Although healthcare budgets are limited, it is not expected blood safety interventions will conform to the generally accepted threshold.

Summary

In summary, this chapter describes the principal elements of blood banking and transfusion medicine. These include regulations and standards that govern the blood supply, donor considerations, blood bank policies and procedures, blood component specifications and indications, and recipient risks. Importantly, the current blood system is considered in the context of an evolving medical system, economic constraints and resource limitations, and novel approaches to blood collection and utilization.

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Hematopoietic Cell Transplantation

37

Gordon L. Phillips II and Hillard M. Lazarus

Introduction

Hematopoietic Cell Transplantation (HCT)

Hematopoietic cell transplantation (HCT) is a lifesaving art and science applied as a curative treatment modality for patients with certain malignant and nonmalignant diseases, predominately of the hematopoietic system. The eradication, treatment, and possible cure of certain of these malignant diseases depend upon, to a variable degree, delivering doses of chemotherapy or chemoradiation therapy that exceed the tolerance of the bone marrow. Infusion of specific hematopoietic cells avoids potentially fatal, irreversible marrow aplasia. Formerly referred to as bone marrow transplantation, high-dose chemotherapy or chemoradiotherapy followed by infusion of hematopoietic stem or progenitor cells allows full lymphohematopoietic reconstitution. Further, in the case of infusion of cells that are obtained from another person, selected immune cells exert an "allogeneic cellular effect" and can eliminate residual malignant cells. HCT also may be used to correct immunodeficiency disorders such as severe combined immunodeficiency syndrome (SCIDS), bone marrow failure states such as severe aplastic anemia, and congenital enzyme deficiencies and to treat, if not cure, various autoimmune diseases, hemoglobinopathies, and other conditions such as multiple sclerosis.

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Definitions for Graft Sources

The hematopoietic stem cell that resides predominately in the bone marrow eventually gives rise to terminally differentiated blood cells, e.g., red cells, white cells, and platelets, over a period of several weeks. Unlike the self-renewing hematopoietic stem cell, these terminally differentiated cells have a relatively short life-span and must be replenished by the bone marrow. Decades ago investigators determined that bone marrow cells transplanted from one animal to another would restore blood production. Subsequently, others demonstrated that these long-term repopulating cells could be obtained from blood (known as blood stem/progenitor cells) after recovery from chemotherapy or after exposure to certain drugs known as hematopoietic growth factors (G-CSF or filgrastim, GM-CSF or sargramostim) or more recently after plerixafor. The latter is a partial agonist of the alpha-chemokine receptor CXCR4 and an allosteric agonist of CXCR7. Its administration also results in mobilization of hematopoietic progenitor/stem cells from marrow into blood (see mobilized blood cell harvesting, below). Finally, umbilical cord blood (UCB) contains a high percentage of such cells, and this product can be collected from the placenta after delivery of a live birth. The vessels of the severed umbilical cord are cannulated using a needle connected to a blood bag, and approximately 75 mL of cord blood is collected using this closed technique. This product is sent to a facility that specializes in the processing and cryopreservation of umbilical cord blood cells for later use. The red cells are depleted, and then the product is cryopreserved and stored in this cord blood bank for future use.

Types of hematopoietic cell grafts:

1. Autologous ("self"). The patient's own blood or bone marrow progenitor/stem cells are collected, cryopreserved, and reinfused after high-dose chemotherapy.
2. Allogeneic (same species but "different genes"). Blood progenitor/stem cells or bone marrow is obtained from another individual, such as a human leukocyte antigen

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(HLA)-matched sibling donor, an HLA-mismatched family donor (and a "half-matched" or haploidentical family donor), or an HLA-matched unrelated donor. UCB cells are an increasingly used graft source even though usually not fully HLA-matched.
3. Syngeneic (identical twin, "same genes"). Marrow or stem cells are obtained from an identical twin.

Hematopoiesis represents a hierarchy of cells with various increments of repopulating capacity. The graft is heterogeneous in nature and contains progenitors for all three lineages (red cells, white cells, and platelets) as well as the hematopoietic progenitor/stem cells. Flow cytometry is performed on samples of the graft to detect the number of CD34+ cells as a frequently used measure for the progenitor/stem cells capable of repopulating the marrow. Despite some limitations, this assay, in general practice, is a useful tool for assessing hematopoietic potency of the graft.

Frequently Used Abbreviations in Hematopoietic Cell Transplantation

1. Many abbreviations that often are used in this field are listed in Table 37.1.

Table 37.1 Commonly used abbreviations in hematopoietic cell transplantation

Abbreviation	Definition
auto	Autologous ("self")
allo	Allogeneic (another person)
ANC	Absolute neutrophil count
HCT	Hematopoietic cell transplant
HSCT	Hematopoietic stem cell transplant
PBSC	Peripheral blood stem cell
DMSO	Dimethyl sulfoxide (cryoprotectant)
HLA	Human leukocyte antigen
GvHD	Graft-versus-host disease
GvL or GvT	Graft-versus-leukemia or graft-versus-tumor
UCB	Umbilical cord blood
RIC	Reduced-intensity conditioning
NMA or NST	Non-myeloablative or non-myeloablative stem cell transplant
t-MDS or t-AML	Therapy-related myelodysplastic syndrome or therapy-related acute myeloid leukemia
DLI	Donor leukocyte infusion
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
TCR	T-cell receptor
SCIDS	Severe combined immunodeficiency syndrome
CFU-GM	Colony-forming unit-granulocyte macrophage
BFU-E	Burst-forming unit-erythroid
TA-TMA	Transplantation-associated thrombotic microangiopathy

Autologous Hematopoietic Cell Transplantation

Overview

1. Using in vitro and in vivo preclinical animal models, many tumors show a steep dose-response curve to cytotoxic therapy. In other words, a small increment in the dose of a chemotherapy drug or radiation increases the number of cancer cells killed by many logs, especially for hematologic malignancies. This principle has been shown to be effective in a number of tumor types. As the dose of chemoradiotherapy is increased, organ toxicities also develop. Notably, the dose of the drug and radiation frequently are limited by marrow injury beyond the capacity of the ability of the various hematopoietic growth factors and/or transfusions and antibiotics to support the patient until hematopoietic recovery returns.

   Bone marrow contains highly proliferative, primitive precursors including hematopoietic progenitor/stem cells that can restore blood production. Using various stimuli such as hematopoietic growth factors (G-CSF or GM-CSF) or other approaches (plerixafor), the bone marrow can be induced to release many of these progenitors/stem cells into the blood; these cells, "mobilized" from the marrow, can be collected and infused and restore marrow function just as infusions of bone marrow can. Mobilized blood or bone marrow progenitor/stem cells (collected directly from the marrow via multiple marrow aspirations) can be obtained from patients (or volunteer donors) before the high-dose therapy is given. These cells can be frozen using sophisticated cryopreservation techniques that utilize cryoprotectant agents such as dimethyl sulfoxide (DMSO) and instruments such as controlled-rate liquid nitrogen freezing chambers. The cellular injury induced by very low temperatures is minimized during the freezing process, and cell viability can be maintained upon thawing. The strategy is to collect and freeze cells, next administer high-dose cytotoxic therapy to the patient, and then thaw and intravenously infuse the previously collected cells which will circulate, home to the marrow, and reconstitute marrow function.
2. Autologous HCT depends entirely upon high-dose chemotherapy or chemoradiation therapy to eliminate the malignancy. In contrast, allogeneic HCT can provide effective antitumor effect by virtue of both administration of high-dose cytotoxic therapy and by the infused donor allogeneic immune cells that can exert an "allogeneic effect" against the tumor, i.e., graft-versus-tumor (GvT) effect (see section on Allogeneic HCT). In both autologous HCT and allogeneic HCT, the "transplanted" hematopoietic progenitor/stem cells are a sophisticated support product that allow the high-dose therapy to be adminis-

Table 37.2 Diseases treated with autologous HCT

Malignant disorders	Nonmalignant disorders
Hodgkin lymphoma	Scleroderma
Non-Hodgkin lymphoma	Systemic lupus erythematosus
Acute myeloid leukemia	Multiple sclerosis
Multiple myeloma	
Germ cell tumors	
Pediatric solid tumors, e.g., neuroblastoma, rhabdomyosarcoma	

tered relatively safely by repopulating chemotherapy-injured bone marrow. Without this cellular support or "rescue," the patient would succumb to the consequences of marrow aplasia, including infection, bleeding, and profound anemia.

3. Autologous HCT procedures also are used as therapy in some severe autoimmune diseases because this treatment can be very immunosuppressive. Table 37.2 lists diseases treated with autologous HCT.

Phases of Autologous HCT

1. *Harvesting*. During harvesting, mobilized blood progenitor/stem cells or bone marrow is collected from the patient who has not been exposed to cytotoxic agents (chemotherapy or radiation) in a number of weeks, hopefully allowing full marrow recovery. These cells give rise to the entire lympho-hematopoietic system. Approximately 1 in 2,000–10,000 nucleated marrow cells has the unique property of self-renewal; the daughter cells produced during cell division give rise to an undifferentiated cell as well as to a more differentiated progenitor cell, the latter beginning the process toward further differentiation and ultimately production of the mature circulating cells. These early progenitor/stem cells are identified as CD34+ by immunophenotyping (as noted above). Some centers also continue to determine hematopoietic-restoring potential by growing cells in in vitro, termed the colony-forming unit assay. This assay is reported as colony-forming unit granulocyte-macrophage (CFU-GM) and burst-forming unit-erythroid (BFU-E) colonies infused per kg patient weight.
   (a) *Bone marrow harvesting* was the traditional method of collecting cells for HCT for the first several decades of transplantation. Marrow is removed from the posterior superior iliac crests with the patient in the prone position under spinal or general anesthesia. In the typical harvest, roughly 1000 mL of marrow is aspirated (10–15 mL/kg patient weight), which requires manually entering the bone marrow space about 100 times. This procedure usually is performed under spinal or general anesthesia in an operating theater. The subject may experience discomfort at the harvest site for several weeks.
   (b) *Mobilized blood cell harvesting* clearly is an easier and simpler way for the clinician to collect progenitor/stem cells when compared to traditional bone marrow harvesting in the operating room. Today, nearly all autologous HCT transplants use hematopoietic cells mobilized from the blood as the graft source.
1. Stem cells and early progenitor cells (see above) normally are found in very low concentrations in the peripheral blood, but stimuli such as marrow recovery after chemotherapy and use of other "mobilizing" agents result in a significant release of these cells into the blood. Chemotherapy injures the marrow and alters the normal equilibrium of stem cells between marrow and blood. The delivery of hematopoietic growth factors, such as granulocyte colony-stimulating factor (G-CSF), alone or after chemotherapy administration, also results in the release of significant numbers of these cells from the marrow. A newer agent, plerixafor, works by disrupting the CXCR4 and SDF-1 lineage between the progenitor and the marrow stromal cell that keeps cells tethered in the marrow microenvironment.
2. The mobilized blood progenitor cells are collected using a large-bore central venous catheter and an apheresis instrument. The desired cells are separated by a centrifugation density gradient and retained, while the unwanted blood cells and plasma are infused back into the patient. This process usually takes about 2–3 h, and about 12–18 l of blood are processed in the procedure. One to three procedures usually are necessary to ensure an adequate yield.
3. *Cryopreservation and reinfusion of cells.* The collected bone marrow or mobilized blood progenitor cells are suspended in a balanced salt solution containing the cryoprotectant dimethyl sulfoxide (DMSO) and are frozen and stored in liquid nitrogen. The dimethyl sulfoxide is protective by helping prevent ice crystallization in the cell during the freezing process. The cells can remain viable after thawing despite being stored in liquid nitrogen (−300 °C temperatures) for at least 5 years and perhaps even longer. After completion of the high-dose chemoradiation therapy regimen (conditioning), the frozen graft is thawed and infused intravenously. While the DMSO can be washed out, it usually is administered with the inoculum and is eliminated via the lungs and kidneys. In patients who have renal insufficiency or cardiac conduction defects such as AL amyloidosis, the DMSO needs to be removed from the graft at the time of thaw in order to avoid hemolysis and cardiac conduction disturbances.
4. *Conditioning (the high-dose chemoradiation therapy preparative regimen).* In the autologous transplant setting, the preparative regimen, which consists of high-dose che-

motherapy and/or total-body irradiation (TBI), is administered first in an attempt to eliminate the malignant disease. In contrast to allogeneic transplantation (see Sect. 3), the use of additional immunosuppressive agents for autologous transplantation is not required to prevent graft rejection or graft-versus-host disease (GvHD). The dose-limiting toxicity of the chemotherapy drugs which are chosen is primarily suppression of the marrow. If the dose-limiting toxicity of the chemotherapy drugs affects other organ systems (i.e., vincristine causes damage to the peripheral nervous system), stem cell infusion will not prevent toxicity. Multiple agents given over several days comprise the conditioning regimen and result in both hematologic and non-hematologic toxic effects (Table 37.3).

5. *HCT.* The transplant involves intravenous infusion of collected bone marrow or blood progenitor cells. The cryopreserved stem cells are thawed and then infused intravenously, similar to a blood transfusion. Through a complex process, cells recognize the marrow microenvironment and "home" from the peripheral circulation to the now-empty marrow space. The cryoprotectant dimethyl sulfoxide (DMSO) is metabolized and eliminated via the lungs and the urinary system (see above).
6. *Recovery.* The infused cellsrequire time to regenerate the marrow, and during this time the patient requires considerable intensive supportive care. The patient is anemic, neutropenic ([absolute neutrophil count] ANC <500/μL), and thrombocytopenic (i.e., pancytopenic). The high-dose chemotherapy additionally has caused breakdown of the normal mucosal barriers in the mouth and gut that increases susceptibility to infectious complications. The mucosal damage to the mouth causes painful ulcerations and limits the ability to eat. Many patients require continuous infusions of opioids. Recovery lasts approximately 2–3 weeks. Visceral organs, also damaged by the chemotherapy and/or radiation, also require time to recover (see below).

(a) *Supportive care during recovery*

1. *Transfusions*. Red blood cell (RBC) transfusions are used to keep the hematocrit over 25% or higher in symptomatic patients. Platelet transfusions are used to keep the platelet count above 10,000–20,000/μL to minimize bleeding; platelet transfusions may be required at a higher threshold in patients who demonstrate a hemorrhagic diathesis. All blood products are externally irradiated to prevent inadvertent engraftment of lymphocytes from the transfused unit that may cause the highly fatal syndrome transfusion-associated GvHD.
2. *Hematopoietic growth factors*
   Use of G-CSF (filgrastim) hastens marrow recovery, particularly WBC count. Such agents usually are begun a few days after the graft has been infused and are continued until a variable but adequate blood neutrophil count is reached.

(b) *Peri-transplant complications*

1. *Infections.* When the neutrophil count drops below 500/μL, there is a marked increased susceptibility to bacterial and fungal infections. The disturbance of the mucosal barrier due to the conditioning regimen and the use of large-bore intravascular access devices add to the patient's risk of experiencing an infection. The longer the neutropenic period, the higher the infectious risk. In most autologous HCT settings, ANC recovery occurs within 2 weeks. The use of antibacterial and antifungal agents in prophylactic fashion, recombinant hematopoietic growth factor (see above), and mobilized peripheral blood progenitor/stem cells (as opposed to use of "steady-state" bone marrow) dramatically has decreased the incidence of severe infections.
   (a) The major infections encountered in autologous HCT transplants are gram-negative and gram-positive bacterial infections and fungal infections, the latter due particularly to *Candida* sp. and *Aspergillus* sp.
   (b) Herpes simplex virus (HSV) infections of the perioral area previously were a common cause of morbidity and rarely mortality, but the use of acyclovir therapy in prophylactic fashion dramatically has decreased this infectious process.
2. *Regimen-related toxicity or organ dysfunction syndromes.*
   Any organ system can be severely damaged by the chemotherapy or chemoradiation therapy administered before the graft is given. The extent of

Table 37.3 Consequences of high-dose chemoradiation therapy in autologous HCT

Hematologic consequences	Other consequences
Marrow aplasia	Mucositis
Immunosuppression, i.e., opportunistic infection	Alopecia
Thrombocytopenia, i.e., serious hemorrhage	Lung injury
	Nausea and vomiting despite antiemetics
	Liver veno-occlusive disease
	Rashes
	Hyponatremia
	Hemorrhagic cystitis
	Cardiomyopathy
	Renal insufficiency

organ damage often is graded and can be mild, moderate, severe, and even fatal. Examples include rash, mucosal barrier to the gastrointestinal and respiratory tracts, and vital organ dysfunction. The use of non-specific supportive care can permit a patient to experience repair. However, there are several specific organ injury syndromes that need to be addressed using specific diagnostic and therapeutic strategies (see below).

3. *Veno-occlusive disease/sinusoidal obstruction syndrome (VOD/SOS) of the liver.* This small-vessel liver disease results from chemotherapy-induced liver injury and is related to the intensity of the preparative regimen. It usually presents within the first 2–3 weeks of the HCT and manifests as tender hepatomegaly, jaundice, and fluid retention. It is reversible in most cases; however, fatalities may result from progressive liver damage, extreme fluid imbalances with respiratory failure, and multisystem organ failure. One of the earliest indicators is impaired flow through the portal vein as assessed using Doppler ultrasound. Defibrotide, a sodium salt of complex single-stranded oligodeoxyribonucleotides derived from porcine mucosal DNA, can be effective therapy. Defibrotide stabilizes endothelial cells by reducing endothelial cell activation and by protecting endothelial cells from further damage, resulting in the restoration of the thrombo-fibrinolytic balance. VOD/SOS is more common in the allogeneic as opposed to autologous transplants (see below).
4. *Idiopathic pneumonia syndrome and diffuse alveolar hemorrhage.* These complications may occur within the first 2 weeks after the transplant but may not appear until later, i.e., 1–2 months after HCT, and may take many months to resolve. Possible causes include lung toxicity from chemotherapy or irradiation and/or viral infections.
5. *Engraftment syndrome and autologous GvHD.* Rarely, some patients may experience significant adverse reactions about the time of hematopoietic recovery. One of these conditions may be termed engraftment syndrome, in which the patient experiences fever, pulmonary vascular leak, rash, and organ dysfunction. The syndrome, which also has been described in association with allogeneic transplants, is thought to reflect an outpouring of endogenous cytokines at a time when blood neutrophil recovery takes place. Onset may be confused with the GvHD.

   An autoimmune syndrome similar to acute GvHD has been reported to occur after autologous HCT and has been termed the "autoaggression" syndrome or autologous GvHD. Its features tend to be milder than classical GvHD and most commonly involve the skin (rarely the gastrointestinal tract, liver, or both) and often are self-limited. Autologous GvHD has been reported to occur both spontaneously and in subjects receiving post-transplant immune modulation with agents such as cyclosporine and interferon or the combination. The mechanism is believed to depend upon the derangement of self-tolerance and depletion of regulatory T cells after the preparative regimen. Disappointingly, the syndrome does not result in lowering posttransplant relapse.
6. *Engraftment*. Engraftment is the period when production of WBCs, RBCs, and platelets by the marrow resumes to minimally acceptable levels that allow reduced or absent support by transfusion and hematopoietic growth factor. The time to a neutrophil count recovery, the usual engraftment standard, has been accelerated with the use of recombinant cytokines/hematopoietic growth factors (e.g., G-CSF) and infusion of mobilized blood progenitor/stem cells rather than bone marrow as the graft source. Median time to a neutrophil count of 500/μL usually is 10–12 days as compared to 18 days or more when steady-state (unstimulated) bone marrow was used without hematopoietic growth factor support. In uncomplicated HCT procedures, the median time to platelet transfusion and RBC transfusion independence usually is 2 weeks.

Outcomes of Autologous Hematopoietic Cell Transplantation

Outcomes depend on the underlying disease and the status of the disease at the time of transplant.

1. When comparing autologous and allogeneic HCT for similar diseases, the following appear to be true:
   (a) Treatment-related mortality is less with autologous transplants (typically <3% during the first 100 days after transplant).
   (b) Relapse rates are higher with autologous transplants (sometimes up to 60–70% of all patients).
2. The major cause of death in autologous HCT is relapse of neoplastic disease. This scenario usually occurs in the 1st or 2nd year after transplant, but relapses and death can occur later.
3. A long-term complication in autologous HCT, rarely observed in allogeneic HCT, is secondary myelodysplas-

tic syndrome (MDS)/acute myeloid leukemia (AML), now categorized by the World Health Organization (WHO) as therapy-related myeloid neoplasms. For a variety of reasons, lymphoma patients appear to be at greatest risk. It remains unclear what treatment is most responsible for the development of treatment-related MDS (t-MDS) or treatment-related acute myeloid leukemia (t-AML), e.g., the initial therapy for the malignancy or the high-dose chemotherapy or chemoradiation therapy. Second cancers also can occur at a higher rate than age-matched controls in the general population, a pattern that may persist for years thereafter.

Advantages and Disadvantages of Autologous HCT

1. *Advantages:*
   (a) A readily available source of blood progenitor/stem cells or bone marrow, i.e., patient, is the donor.
   (b) Low treatment-related mortality.
      1. The mortality rate 100 days after the HCT presently is approximately 0.5–1.0%.
      2. The 100-day mortality rate for allogeneic HCT often is 25% and can be as high as 40% in some high-risk groups. Use of lesser intensive conditioning regimens has reduced the early mortality rates observed with full-intensive (myeloablative) conditioning.
   (c) Relatively low toxicity (compared to allogeneic transplant).
      1. Unlike allogeneic HCT, no immunologic barriers must be crossed with use of an autologous HCT, and there is no need for immunosuppressive medication to be administered after the transplant.
      2. As there is no GvHD, immunosuppressive medications given after allogeneic HCT, such as cyclosporine and tacrolimus, are unnecessary, and hence posttransplant infectious complications are fewer. Rarely, some patients experience engraftment syndrome and autologous GvHD (see below).
      3. Graft rejection (failure of the infused cells to engraft) is rare. Some patients may not have full recovery of blood counts and may have low grade, chronic anemia, leukopenia, thrombocytopenia, or the combination (pancytopenia). Most affected patients function at normal levels, but some experience problems relating to cytopenias and require continued supportive care.
      4. Chronic visceral organ dysfunction. Some patients have persistent organ damage due to therapy that may be lifelong such as mild to moderate cardiac, hepatic, and renal dysfunction.
2. *Disadvantages:*
   (a) Tumor contamination.
      1. The cryopreserved graft may contain viable tumor cells, but the clinical significance is not clear. Gene-marking studies have shown that rarely these autologous tumor cells can contribute to clinical relapse, usually in sites of prior neoplastic deposits.
      2. When infused into the patient, these tumor cells may contribute to relapse after HCT.
   (b) Lack of an allogeneic antitumor effect.
   There is no graft-versus-tumor (GvT) effect, a major feature with allogeneic HCT (see Sect. 3: "Benefit of donor T cells"). The tumor cell killing effect depends entirely on the transplant conditioning regimen (high-dose chemotherapy or chemoradiation therapy).
   (c) Rare problems such as "engraftment syndrome" and "autologous GvHD" can occur. The former is a poorly understood condition characterized by high fever in the peri-engraftment period likely due to an outpouring of endogenous cytokines in response to the stresses associated with cytopenias and toxic agent exposure. The latter is a syndrome that can occur in which the infused autologous cells appear to attack visceral organs (skin and GI tract most commonly, liver rarely) in a manner similar to that observed in allogeneic transplant recipients. Both conditions usually respond to short courses of high-dose corticosteroids.

Allogeneic Hematopoietic Cell Transplantation

Overview

Allogeneic HCT is the administration of chemotherapy or chemoradiation therapy to eliminate a neoplastic or nonneoplastic disease followed by infusion of normal donor-derived bone marrow or blood progenitor cells. The donor graft is obtained from an HLA-matched or HLA-mismatched family member (usually a sibling) or an unrelated donor such as an adult matched unrelated donor (MUD) or an umbilical cord blood (UCB) graft. Recently, the use of haploidentical family member donors has been an emerging graft source (see below). As a result, most, if not quite all, patients have potential donors. In recent years, sophisticated research using a dog animal model demonstrated that employment of a somewhat less intensive cytotoxic agent therapy could induce a sufficient immunosuppression effect to facilitate the engraftment of allogeneic cells. In contrast to a myeloablative or full-intensity conditioning HCT, this less

intensity conditioning strategy has been termed reduced-intensity conditioning (RIC) or non-myeloablative (NMA) conditioning. The advantage to this approach is that much older patients (usually >60 years) could undergo HCT with relatively greater safety. Also, patients who are otherwise good HCT candidates but ineligible for a myeloablative conditioning HCT due to one or more comorbid illnesses (cardiomyopathy, lung dysfunction, renal insufficiency, etc.) could be candidates for a RIC or NMA conditioning HCT as the likelihood and extent of organ injury incurred are reduced with use of a less intensive cytotoxic therapy approach. The engrafted cells still act as effector cells and mediate GvT effects. Several investigators have provided definitions for what constitutes full-intensity (myeloablative), reduced-intensity, or non-myeloablative conditioning (see the citation [1]).

Indications for Allogeneic HCT

1. *Malignancy*
   (a) The most common use of allogeneic HCT is for hematologic malignancies, such as acute myeloid leukemia, acute lymphoblastic leukemia, myelodysplastic syndrome, chronic lymphocytic leukemia, chronic myeloid leukemia, myeloproliferative neoplasms, Hodgkin lymphoma, non-Hodgkin lymphoma, and multiple myeloma.
   (b) The curative potential of allogeneic HCT relies on three concepts:
      1. Higher doses of chemotherapy and chemoradiation therapy may overcome the resistance to standard chemotherapy of some aggressive tumor cells as well as completely ablate or eliminate host bone marrow cells. In this setting, the infusion of donor hematopoietic progenitor cells after high-dose chemotherapy or chemoradiation therapy functions as a "biologic marrow rescue." The use of a full-intensity regimen, while associated with more toxicity to the recipient, is associated with lower relapse rates due to the more potent treatment. The corollary of lower toxicity but higher relapse rates is observed with use of RIC and NMA conditioning.
      2. Infusion of normal donor marrow or mobilized blood progenitor/stem cells after high-dose chemotherapy re-establishes normal hematopoiesis.
      3. The donor cells eliminate the patient's tumor through immunologic mechanisms, the GvT effect; relapse rates will be lower than in autologous HCT because viable tumor cells that remain despite high-dose chemotherapy can be eradicated by this cellular effect.
   (c) The decision as to when to proceed with allogeneic HCT varies by disease and stage.
      1. For some acute myeloid leukemia (AML) patients such as those with unfavorable cytogenetics or molecular markers, allogeneic HCT is the only curative therapy and is therefore recommended for all patients under age 55 years.
      2. In acute lymphocytic leukemia, many patients (especially children) are cured with conventional chemotherapy. Therefore, transplantation is recommended only for patients who relapse after standard therapy or patients who have features that indicate they are at a high risk for relapse.
   (d) The cure rate of allogeneic HCT after relapse is lower than during remission, and not all patients can achieve another remission after relapse. Allogeneic HCT is offered as an option in relapse only under well-defined circumstances such as in the context of a clinical trial and then infrequently.
2. *Bone marrow failure.* Allogeneic HCT often remains the treatment of choice for patients with severe aplastic anemia, particularly those who do not respond to immunosuppressive therapy.
3. *Inherited disorders*
   (a) *Hematopoiesis.* This category consists of a variety of rare congenital disorders. Although uncommon conditions, transplantation in this group demonstrates that any disease involving a hematopoietic stem cell or its progeny can be cured through an allogeneic HCT transplant. These diseases are caused by the developmental absence or abnormality of a specific lineage of cells derived from the lymphohematopoietic stem cell.
      1. *Immunodeficiency diseases*
         (a) Severe combined immunodeficiency is a heterogeneous group of lethal disorders of T and B lymphocytes that predispose the patient to life-threatening infections.
         (b) Wiskott-Aldrich syndrome is an X-linked recessive disorder characterized by T-cell immunodeficiency.
         (c) Osteopetrosis is a rare disease that is a result of dysfunctional osteoclasts (specialized macrophages derived from the marrow stem cell).
      2. *Hemoglobinopathies*
         (a) This category includes severe disorders of hemoglobin synthesis, such as sickle cell disease and thalassemia.
         (b) In European countries, allogeneic HCT transplant is more frequently used for these diseases than in the United States.
   (b) *Storage diseases.* This disease category includes a number of rare disorders in which an enzyme defi-

ciency leads to an accumulation of toxic endogenous products normally degraded and eliminated by the body macrophages. The buildup of these derivatives leads to a wide range of irreversible neurologic disorders, such as Hurler syndrome, Hunter syndrome, and mucopolysaccharidoses. HCT provides the deficient enzyme in blood cells that can overcome the target organ's enzyme deficiency. The transplant usually must be completed before the patient becomes symptomatic and the damage irreversible.

Immunologic Aspects of Allogeneic HCT

1. *Human leukocyte antigen (HLA) system*
   1. A donor for the transplant is first determined by the matching of HLA proteins, transmembrane proteins that function normally in antigen recognition of foreign agents (e.g., viruses, bacteria). This system is important for immunologic recognition of "nonself," i.e., foreign tissues. They bind to protein fragments and present these fragments (antigens) to T cells, which results in T-cell activation via the T-cell receptor (TCR).
   2. HLA proteins are encoded by genes of the major histocompatibility complex (MHC) on chromosome 6. A set of four (4) molecules on each chromosome (the A, B, C, and D complex DR/DP/DQ) defines a haplotype. Humans inherit a separate haplotype from each parent, and each haplotype is codominantly expressed on the cell surface, i.e., everyone has a pair of these four molecules. Hence, the "optimal match" situation for an allogeneic HCT is when there is matching at all four pairs of molecules, to which people refer as an "8/8 match." In recent years, the extent of matching has been widened, and many centers target a "10/10 match" or "12/12 match." Lesser degrees of matching are associated with worse patient outcomes.
   3. Each HLA antigen is polymorphic, with hundreds of different alleles for each of the three antigens, making the typing process complex.
      (a) The A, B, and C antigens, known as the class I antigens, are usually expressed on all cell types except erythrocytes. The C antigen is located physically between A and B; if A and B are matched, C also most likely would be inherited. Sometimes separate typing for the C antigen is not performed, except in URD HCT in which C mismatching may profoundly affect overall survival and graft-versus-host disease (GvHD) (see below).
      (b) The class II antigens, or DR/DP/DQ antigens, are usually limited to B cells, monocytes, dendritic cells, and some activated T cells.
      (c) Antigen disparity can be at the level of minor histocompatibility antigens (miHA), inherited differently. In the setting of an MHC-matched but miHA-mismatched HCT, GvHD can occur. Some miHAs such as HA-1, HA-2, HB-1, and BCL2A1 are primarily found on hematopoietic cells, whereas some others such as the H-Y antigens, HA-3, HA-8, and UGT2B17 are ubiquitous.
      (d) The severity of acute GvHD is closely related to the degree of MHC mismatch.
2. *Finding a suitable donor*
   The approach to identifying a donor has changed greatly over the years and reflects both advances in histocompatibility understanding and testing and significant expansion in donor registries, umbilical cord blood banks, and the processes to identify alternative donor graft sources.

(a) The most desirable source of hematopoietic progenitor/stem cells is a "full" HLA-matched sibling, i.e., "12/12" matching as described above for the HLA-A, HLA-B, HLA-C, and HLA-D complex pairs. As the haplotypes are inherited in codominant Mendelian fashion, each full sibling has a 25% chance of matching. Sometimes other close relatives are matches, especially in cases of consanguinity. Identical (syngeneic) twins are another situation for which a transplant can be performed.

(b) If no immediate family members match, there are registries of normal adult donors who have agreed to donate blood and marrow to patients who need transplants. These unrelated donors are less likely to match for miHA, which can cause immunologic reactions between donor and recipient. It is estimated currently that nearly 30 million altruistic adults are in these registries worldwide.

(c) Umbilical cord blood (UCB) also is an emerging graft source. For UCB transplantation, often only three of the HLA pairs are evaluated (the HLA-C loci are omitted), leading to only six (6) loci to be matched. Although this type of donor product often is matched only at 4/6 loci, the cells are immunologically naïve and are less likely to be associated with GvHD. Specifically, for a given degree of HLA matching, the incidence of GvHD is reduced compared to a MUD adult donor, yet there is a retained graft-versus-malignancy (GvT) effect. Obviously, better matched is desired, if such is possible.

(d) Partially matched related or haploidentical ("haplo") transplants are being performed in greatly increased frequency. The potential for using this graft source results when each donor has inherited the same genetic material (parental chromosome) from one but not both parents.

The key advantage is that almost all patients will have a haploidentical donor if the search is broadened to include their nuclear or extended family, e.g., parent, sibling, child, and parent. Some decades ago, these HCT procedures were attempted using techniques designed for matched family members and as a result were largely unsuccessful due to increased rates of graft rejection or GvHD. Several groups pursued this approach predominately using various methods of T-cell depletion, either ex vivo or in vivo. In the ex vivo setting, donor T cells are inactivated or removed from the graft mechanically or chemically. For in vivo T-cell depletion, the recipient is given high-dose posttransplantation cyclophosphamide IV (*PTCY*) several days after graft infusion in order to eliminate the infused alloreactive donor T cells. This maneuver, however, spares the donor hematopoietic stem cells that possess high concentrations of aldehyde dehydrogenase to resist cyclophosphamide injury. A number of centers have begun to adopt this approach, alone or in combination with other posttransplant immunosuppressive agents, and this strategy appears to be increasing in frequency.

Moreover, haploidentical transplants produce a situation in which there are multiple potential donors. Algorithms to guide and facilitate donor selection are in use and vary according to the method of transplant. Patients also may have preformed donor-specific antibodies such as may occur in multiparous woman or in transfusion recipients. Such antibodies can prevent engraftment, and use of a donor/patient crossmatch test needs to be performed during the selection process. Other issues include the presence of additional, fortuitous sharing of antigens on the non-inherited chromosome.

3. *Benefits of infusing donor T cells.* The harvesting of hematopoietic progenitors/stem cells, either from the mobilized donor peripheral blood or from the donor marrow, contains a mixture of mature WBCs with progenitors and some stem cells in the collection bag. Mature T lymphocytes are the principal effectors of cell-mediated immunity; many of the therapeutic benefits and toxicities of allogeneic HCT transplant are derived from immunologic reactions between donor T cells and recipient (host) cells.
   1. *Engraftment*. Donor T cells appear to facilitate engraftment. These cells, in part, eliminate remaining elements of host immune cells that survive the cytotoxic effects of the conditioning regimen and might reject the donor graft. Depletion of donor T cells before allogeneic HCT transplant significantly increases the risk of graft failure. This effect is most pronounced in the setting of non-myeloablative transplant (and to a lesser extent in reduced conditioning transplant), in which the lesser-intensive conditioning may not turn off the host immune system.
   2. *Graft-versus-leukemia (GvL) effect.* Most allogeneic HCT are performed for malignancies that do not completely respond, or will relapse despite exposure, to conventional doses of chemoradiotherapy. Donor T cells are capable of recognizing and eliminating residual malignant cells in the host. Both CD4+ and CD8+ cells contribute to this graft-versus-leukemia effect in animal models, although the relative importance of specific subsets in clinical transplantation is not yet clear. Depletion of most donor T cells increases the risk of relapse in many situations. If a patient's neoplastic disease recurs after HCT, sometimes infusion of additional donor leukocytes (known as donor leukocyte infusion or DLI) can again eradicate the malignancy. Use of DLI, unfortunately, has been associated with an increase in the likelihood of the recipient developing GvHD, which can be fatal.
   3. *Immunologic reconstitution.* The thymus involutes with increasing age. Reconstitution of the immune system is slower after allogeneic than after autologous HCT. T cells are the last of the immune cells to recover. T-cell depletion further retards the pace of T-cell reconstitution, making recipients even more prone to reactivation of latent viral infections, including cytomegalovirus (CMV), adenovirus (Bk), varicella-zoster virus (VZV), and others.
4. Disadvantages of infusing donor T cells. GvHD is the greatest disadvantage from the presence of donor T cells.
   (a) *Overview of graft-versus-host disease (GvHD)*
   Described more than 50 years ago by Billingham and co-workers, GvHD is a complex and intricate syndrome resulting from a consequence of interactions between the donor and host innate and adaptive immune responses. This interaction of donor T cells with antigen-presenting cells (APCs), essentially the "sensors" for acute GvHD, is regulated positively or negatively by numerous cytokines, chemokines, and immune cell subsets.
   1. Three conditions are necessary for the development of GvHD:
      (a) An immunocompetent graft (i.e., one containing donor T cells)
      (b) Histocompatibility (minor or major) differences between donor and recipient
      (c) A recipient who cannot mount an immune response to the graft
   2. The chemotherapy or chemoradiotherapy used to condition HCT recipients and the large number of T cells within the graft combine to make allogeneic HCT the most common setting for acute GvHD. Without addi-

Table 37.4 Procedures associated with a high risk of GvHD

Hematopoietic cell transplantation	Patients receiving on GvHD prophylaxis
	Older patient age
	Recipients of HLA-nonidentical grafts
	Recipients of grafts obtained from allosensitized donors
Solid organ transplant	Recipients of organs containing lymphoid tissue, i.e., small bowel transplant
Transfusion of unirradiated blood products	Neonates and fetuses
	Patients with congenital immunodeficiency syndromes
	Patients receiving immunosuppressive chemoradiation therapy
	Patients receiving directed blood donations from partially HLA-identical, HLA-homozygous donors

GvHD graft-versus-host disease, *HLA* human leukocyte antigen

tional immunosuppression after administration of the donor graft, more than 90% of allogeneic HCT recipients would develop significant GvHD, even from HLA-identical siblings (in this case due to differences in minor histocompatibility antigens between siblings).

3. Other individuals at risk for GvHD (Table 37.4) are recipients of blood transfusions that contain WBCs (including lymphocytes). Normal individuals who are heterozygous for HLA proteins will not reject lymphocytes transfused from a donor who is homozygous for one of the recipient's haplotypes. If the donor lymphocytes that are not rejected recognize HLA antigens of the host's other haplotype, GvHD may develop. This syndrome is known as transfusion-associated GvHD and is more common in "inbred populations" who share many antigens. GvHD also may develop in patients who received directed blood donation but are not otherwise compromised (e.g., transfusion from an HLA-homozygous mother to a heterozygous child). External irradiation of blood products prevents lymphocytes from proliferating; therefore, all immunocompromised patients must receive irradiated blood products.
4. Clinical presentation. In general, there are two types of GvHD—acute and chronic—although an "overlap" form exists that has features of both. Due to the differing times of onset (usually), traditionally GvHD before 100 days after transplant was denoted as acute, and onset later was designated as chronic. This categorization, however, was deemed simplistic, and now these types are distinguished clinically.

   In brief, acute GvHD usually occurs weeks after transplant and resembles an inflammatory disorder primarily affecting the epithelial cell component of three target organs: the skin, gastrointestinal tract, and liver. The effector cell appears to be the T-cell lymphocytes contained within the graft. Controversially, some other organs are involved infrequently. Late-onset acute GvHD appears to be more common in patients who received RIC or non-myeloablative conditioning (as opposed to myeloablative conditioning). This syndrome may have a worse prognosis.

   In contrast, chronic GvHD often occurs months after transplant, often but not invariably preceded by acute GvHD. This syndrome is a multisystem disorder that resembles, to a degree, certain autoimmune diseases, especially scleroderma as it is characterized by both inflammatory and fibrotic components. The effector cell appears to be mediated by several cell types including a T cell that arose from the donor stem and progenitor cells in the graft that were "educated" by a different (the host) immune system, i.e., a situation of immune dysregulation. In contrast to acute GvHD, there are reports of chronic GvHD involving virtually any organ, although the skin, eye, liver, lung, joints, and nerves are often involved (see below).
5. Once established, GvHD is difficult to treat, and severe cases are usually fatal. The mainstay of therapy is high-dose corticosteroids. While there have been a myriad of other therapies that have been tried, the success has been variable, and these approaches are not a standard of care.
   (b) *Pathophysiology of acute GvHD*. The pathophysiology of acute GvHD can be considered using a framework of model that breaks down this syndrome mechanistically into three sequential phases. While clearly not a stepwise process occurring in discrete stages, this model is conceptually useful.
      1. *Phase one.* The earliest phase of acute GvHD starts before the donor cells are infused. The transplant conditioning regimen damages and activates host tissues (e.g., intestinal mucosa, liver) that secrete inflammatory cytokines that can upregulate adhesion molecules and MHC antigens. This sequence enhances recognition of host allogeneic antigens by mature donor T cells contained in the graft.
      2. *Phase two.* During this phase of acute GvHD, host antigen-presenting cells interact with and activate donor T cells that then proliferate and differentiate. When donor and recipient are not MHC identical, donor T cells can recognize host MHC molecules as foreign, and the resultant graft-versus-host reaction can be dramatic. In the setting of MHC-identical recipient and donor, different peptides bound to the MHC are recognized by the T cell and its T-cell receptor

(TCR). The T cells secrete interleukin-2 (IL-2) and interferon-γ (type 1 cytokines) as critical mediators of acute GvHD.

3. *Phase three.* This component is the effector phase of acute GvHD. In addition to the activated donor T cells, mononuclear phagocytes, primed by cytokines during phases one and two above, play an important role in this third phase of acute GvHD. Monocytes receive a second, triggering signal to secrete the inflammatory cytokines tumor necrosis factor-α (TNF- α), interleukin-l (IL-1), and IL-6. Toll-like receptors (TLR) recognize pathogen-associated molecular patterns (such as lipopolysaccharide, i.e., endotoxin) released during conditioning and initiate cellular signaling pathways leading to secretion of these proinflammatory cytokines. Lipopolysaccharide (endotoxin) from conditioning regimen-damaged intestinal mucosa stimulates gut-associated lymphocytes and macrophages. TNF-α may directly cause tissue injury by inducing either necrosis or apoptosis (programmed cell death) of target cells. Thus, the induction of inflammatory cytokines may synergize with the cellular damage caused by cytotoxic T cells and natural killer cells, resulting in the amplification of local tissue injury.

(c) *Clinical grading of acute GvHD.* This syndrome, in some ways a complex immune response that has gone awry, is graded 0 through IV (Glücksberg grade) or A through D (IBMTR grade) based on organs involved and extent (stage) of involvement. Patient outcome significantly deteriorates with increasing grade of acute GvHD. Table 37.5 illustrates the complex grading system used to score the extent of acute GvHD. The two most commonly used scales, the IBMTR and the Glücksberg, are shown. In each case, a value is given for the degree of involvement for the three major affected organs: the skin, GI (gastrointestinal) tract, and liver. If a patient has several organs involved, or one organ that is severely involved, the overall score or "grade" is higher, which predicts a worse patient outcome. The IBMTR grading scale scores acute GvHD from the lowest ("A") to the highest ("D") grade. The Glücksberg grading employs a numeric system from "I" as the lowest to "IV" for the highest grade.

Table 37.5 Clinical grading of acute GvHD

IBMTR grade	Glücksberg grade	Skin	GI	Liver
A	I	1	0	0
B	I	2	0	0
B	II	0–2	1	0–1
B	II	0–2	0–1	1
C	II	3	1	0–1
C	II	3	0–1	1
C	II	3	0	0
B	III	0–2	2	0–2
B	III	0–2	0–2	2
C	III	0–3	0–3	2–3
C	III	3	2–3	0–3
D	III	0–3	0–3	4
D	IV	0–3	4	0–4
D	IV	4	0–4	0–4

Adopted from Cahn et al. [2]

One group [3] developed a validated serum biomarker panel for acute GvHD. In the initial report, four proteins (interleukin-2 receptor-alpha, tumor necrosis factor receptor-1, interleukin-8, and hepatocyte growth factor) optimally discriminated patients with and without acute GvHD. This panel of biomarkers can confirm the diagnosis of GvHD at onset of clinical symptoms and provide prognostic information independent of GvHD severity.

(d) *Prevention of acute GvHD.* All allogeneic HCT recipients are treated with immunosuppressive agents before graft infusion as prophylaxis against GvHD. Usually at least two classes of drugs are used together.

1. *Tacrolimus (FK-506) or cyclosporine, so-called calcineurin inhibitors.* These compounds are extremely immunosuppressive and block TCR signaling pathways; only one (not both) is used. These agents also are toxic drugs, particularly to the kidneys (which additionally may be weakened by use of antibiotics). Treatment prophylaxis usually lasts at least 6 months after transplant.
2. *Methotrexate (MTX).* A dihydrofolate reductase inhibitor, MTX, inhibits expansion of all actively dividing cells. MTX is most effective when given as several doses (short course; not continued) within the first 2 weeks after transplant, the time of maximal donor T-cell response to host antigens. MTX slows expansion of donor T-cell clones but also that of donor hematopoietic stem cells, and engraftment often is slowed by several days when MTX is used. Mucositis also is increased with its use.
3. *Corticosteroids*. Rarely are these agents given as prophylaxis. Prednisone and prednisolone possess potent immunosuppressive properties among many other side effects and can induce apoptosis and lyse lymphocytes. They also inhibit production of inflammatory cytokines that mediate GvHD. Use of this group of drugs usually is reserved for treatment of GvHD that develops despite prophylaxis.

4. *Mycophenolic acid.* This agent usually administered as the prodrug *mycophenolate mofetil* (MMF; trade name CellCept) to improve oral bioavailability is an immunosuppressant drug used to prevent rejection in organ transplantation. This agent inhibits an enzyme needed for the growth of T cells and B cells. This agent more frequently is used instead of methotrexate in the course of GvHD prophylaxis for RIC and non-myeloablative conditioning and UCB and haploidentical transplants.
5. *Sirolimus.* This drug, which can be given by mouth and IV, also has been known as rapamycin. It acts as an immunosuppressant by inhibiting activation of T and B cells by reducing the production of interleukin-2. The most common adverse reactions include peripheral edema, hypercholesterolemia, GI upset, renal dysfunction, marrow suppression, and drug interactions.
6. *PTCY* (posttransplantation cyclophosphamide). Use of this agent falls under the category of in vivo T-cell depletion. The recipient already will have received the conditioning regimen and infusion of the graft before initiation of the high-dose cyclophosphamide IV, which usually is administered the 3rd and 4th days after graft infusion. At this time, the alloreactive donor T cells in the graft are beginning to recognize the host and proliferate and mount an attack, as such they are killed by this therapy. The donor hematopoietic stem cells possess high concentrations of aldehyde dehydrogenase and usually resist significant cyclophosphamide injury. Hematopoietic recovery usually is slower, and relapse rates after transplant may be higher.
7. *T-cell depletion (in vitro).* In this situation, the donor graft is manipulated in the laboratory to remove T cells that can mediate the GvHD reaction. Cells can be eliminated using physical, chemical, or immunologic means. Removal of T-cell populations may be associated with reduction or elimination of T cells that facilitate engraftment, and as a result, the risk of engraftment failure or slower hematopoietic recovery is greater. Further, loss of T cells may be associated with higher rates of relapse and opportunistic infection.

(e) *Treatment of acute GvHD.* Depending on the graft source and other factors, one-third to two-thirds of patients will develop GvHD despite prophylaxis with immunosuppressive agents. If involvement is confined only to the skin, i.e., grade I, usually only topical corticosteroid therapy is applied to the skin or lower doses of prednisone (≤0.5–1.0 mg/kg/day). If involvement is more extensive in the skin or the GI tract, liver, or combinations are involved, higher doses of prednisone 2 mg/kg/day are administered; higher doses of corticosteroids are considered more toxic but not more effective. Often patients respond and the prednisone dose is slowly tapered off. Patients in whom acute GvHD does not respond to prednisone are considered "steroid-refractory." When the response to frontline corticosteroids for acute GvHD is minimal, delayed, or absent, or when tapering of such therapy is associated with the inability to maintain control of acute GvHD, this situation is defined as steroid-resistant acute GvHD. There is no standard treatment although many agents are used clinically. Approaches tried often include (alone or in combination) ATG, tumor necrosis factor inhibitors (infliximab and etanercept), pentostatin, MMF (CellCept), denileukin diftitox, and anti-IL-2 receptor (basiliximab)-, anti-IL-6 receptor (tocilizumab)-, anti-CD20 (rituximab)-, and anti-CD52 (alemtuzumab)-targeted therapies. Further, extracorporeal photopheresis (ECP) and also infusion of mesenchymal stromal cells (MSCs) are employed, among other approaches. This situation remains one of intense basic and clinical investigations. These patients usually succumb either to progressive organ dysfunction due to GvHD (despite the addition of other potent immunosuppressants), or they develop fatal opportunistic infections.

(f) *Chronic GvHD.* For a variety of reason, this syndrome is less well understood than acute GvHD and cannot reliably be prevented. It can evolve after acute GvHD (progressive) or develop in the absence of acute GvHD (de novo). Some data suggest that the prophylactic use of PTCY or ATG can lower the incidence and severity of chronic GvHD. This syndrome is most difficult to treat for pragmatic reasons as it usually develops after a patient has been discharged from the hospital. Further, the pathobiology is complex and involves a number of cell types in addition to T cells, and it is just being better understood. This condition may affect 30–50% of allograft recipients and is the leading cause of non-relapse mortality. Chronic GvHD accounts for the death of 25% of persons surviving at 2 years after transplant and 11% of those surviving 5 years after transplant.

A variety of agents are utilized as there is no single preferred therapy. Patients are at markedly increased risk of opportunistic infection despite having adequate neutrophil blood counts; fatal infection due to fungal, viral, and bacterial organisms is not uncommon. Infection accounts for 60–80% of death in affected patients. In some patients, a state of "tolerance" develops after years, and chronic GvHD ultimately may subside. Median duration of therapy is 2–3 years after transplant, and 15% of patients still

require treatment by 7 years after transplant. Often times, non-transplant physicians in the community may encounter affected patients as these subjects no longer are under intense scrutiny by the transplant physicians given the length of time after transplant.

Some additional features of chronic GvHD include the following: (1) an increasing incidence related to a number of factors such as the greater reliance on the use of mobilized blood progenitor/stem cells (vs. steady-state bone marrow) as the graft source; (2) greater likelihood to affect older patients; (3) worse prognosis with more severe forms, especially when resistant to first-line therapy with glucocorticoids (as below); (4) with more severe forms, the beneficial effect of the "graft-versus-tumor" reaction is negated by the increased mortality rate; (5) a profound effect of not only increased mortality but also reduced patient quality of life with all but the mildest forms; and (6) inadequate therapies, as indicated below.

Clinical grading is as per the NIH classification. The reader is referred to the comprehensive organ scoring tables [4]. More severe forms are associated with a worse outcome.

Recently, incorporation of anti-thymocyte globulin (ATG) into the conditioning regimen and the use of PTCY (posttransplantation cyclophosphamide) have been shown to be beneficial as prophylaxis strategies for reducing the incidence of chronic GvHD. Overall, however, therapy for chronic GvHD has not advanced much, and glucocorticoids (with or without a calcineurin inhibitor such as tacrolimus) are used as treatment. A wide number of other agents and techniques have been tested, albeit usually not in controlled, randomized clinical trials. Apheresis with ECP and a number of new agents such as low-dose interleukin-2 (IL-2), ruxolitinib, and ibrutinib, among others, appear to show promise and are being tested for response and tolerability.

Phases of Allogeneic Hematopoietic Cell Transplantation

1. *Conditioning.* Before HCT, the patient receives high-dose chemotherapy or chemoradiation therapy, the twofold purpose to eliminate malignant and prevent the host from rejecting the graft.
   (a) *Cytoreduction.* While chemotherapy and radiation therapy kill tumor cells, this treatment also eliminates the patient's normal or abnormal stem cells. The most common preparative regimens are high-dose cyclophosphamide and total-body irradiation (TBI) or high doses of busulfan and cyclophosphamide. These combinations have broad antitumor and immunosuppressive activity. Again, the use of more intensive (myeloablative or full-intensity) conditioning is associated with greater antitumor effect and lower relapse rates, often at the expense of greater toxicity, when compared to RIC and NMA conditioning.
   (b) *Immunosuppression.* Conditioning also is immunosuppressive. To allow engraftment of the new hematopoietic progenitor and stem cells, the patient's endogenous lymphocytes must be eradicated to prevent the rejection of the new, donor cells.
2. *Transplant.* The donor graft cells are infused intravenously as with a blood transfusion. The S/progenitor cells "traffic" or "home" via a complex process to the appropriate places in the bone marrow where they take up residence and proliferate.
3. *Recovery.* This period of intensive supportive care is similar to the recovery period in autologous HCT. High-dose chemotherapy and radiation therapy eliminate normal marrow function as well as cause potentially reversible damage to many organs, including the skin, GI tract, lungs, liver, kidneys, and other organs. Many of these organ injuries are similar to that encountered in the course of an autologous HCT.
   (a) *Aggressive supportive care* is crucial for long-term success as most toxicities are reversible, i.e., sophisticated support provides the time necessary for host organ regeneration and recovery. In particular, vigorous use of antibacterial, antiviral, and antifungal agents is given as prophylaxis as well as to treat active infections.
      1. *Transfusions.* There is an obligate period of aplasia while awaiting marrow recovery, during which time patients require RBC and platelet transfusions. Prolonged neutropenia predisposes patients to a multitude of bacterial infections, and routine granulocyte transfusions are impractical and reserved for special circumstances.
      2. *Cytokine and hematopoietic growth factor support.* Hematopoietic growth factors (e.g., G-CSF) usually are given to hasten neutrophil recovery after HCT, although these agents are used less commonly in allograft than with autologous transplants.
   (b) *Complications during recovery.*
   In addition to acute and chronic GvHD (discussed above), other complications can occur, some similar to those seen after autologous HCT.
      1. *Infections.* As patients are neutropenic early after transplant and are receiving potent immunosuppressive GvHD prophylaxis agents, they are susceptible to a variety of infections. Additionally, much earlier in their disease course, patients may

have required broad-spectrum antibiotics to prevent or treat infection that subsequently could lead to microorganism resistance or result in internal organ injury when rechallenged with antibiotics. In the course of HCT, bacterial infections may develop during the neutropenic period. Further, a superinfection with bacteria may develop later as patients are receiving immunosuppressants; the slow immune reconstitution and defects in cellular and humoral immunity (hypogammaglobulinemia) also contribute. The lack of a fully functioning immune system often leads to reactivation or active viral infections and unusual or opportunistic infections and fungal infections. In addition to these deficiencies, the normal barriers that prevent entry of microorganisms (e.g., skin, GI tract, respiratory epithelium, etc.) have been damaged. Prompt recognition and institution of various antibacterial agents and the judicious use of antiviral and antifungal agents greatly lessen the risk of these infections.

(a) *Bacterial infections.* During the period of neutropenia (blood granulocyte count <500/μL), common bacterial infections include gram-positive cocci (usually *Staphylococcus epidermidis* from central venous catheter devices and *Staphylococcus aureus*) and gram-negative rods (*E. coli*, *Klebsiella* sp., and occasionally *Pseudomonas* sp. from the GI tract and respiratory tract).

(b) *Viral infections.* Several viruses including CMV, HSV, VZV, adenovirus, and respiratory syncytial virus can be fatal after HCT. Serious damage from CMV and HSV can be prevented via prophylactic antiviral therapy (e.g., ganciclovir, acyclovir). Other viruses are much more difficult to treat. Viral infections occur more frequently after allogeneic HCT than after autologous HCT.

(c) *Fungal infections.* Candidiasis and aspergillosis are less common but often fatal infections in patients after HCT. Strategies for prophylaxis of candidiasis with fluconazole therapy have been beneficial but ineffective for aspergillosis; agents with significant activity against this pathogen include voriconazole, micafungin, and posaconazole, among others. One of these agents should be utilized when patients are receiving corticosteroids to prevent or treat active GvHD.

2. *GI tract injury.* Both conditioning and GvHD damage the GI tract. Use of multiple antibiotics during HCT also changes bacterial flora (e.g., *Clostridium difficile* enterocolitis). Parenteral nutrition frequently is administered after allogeneic HCT, but the data supporting this routine practice are not overly compelling.
3. *Liver dysfunction.* Both hepatic veno-occlusive disease (also known as sinusoidal obstruction syndrome) and GvHD (acute and chronic) cause liver disease. Veno-occlusive disease usually results from liver injury induced by the conditioning regimen which most often occurs within the first 2–3 weeks after both allogeneic and autologous HCT. A new agent defibrotide appears to be extremely effective in severe cases of hepatic veno-occlusive disease. Many antibiotic medications such as voriconazole also can be associated with liver injury.
4. *Lung injury.* Both autologous and allogeneic HCT may result in damage to the lungs. Viral, fungal, and bacterial infections are common, but pathogens are identified in only 50% of cases; the remaining cases are probably immunologic or injury from the preparative regimen, e.g., interstitial pneumonia, idiopathic pneumonia syndrome, and diffuse alveolar hemorrhage. The lungs are a target of both acute and chronic GvHD, and patients are at risk for a progressive, serious lung injury known as the bronchiolitis obliterans syndrome.
5. *Immune system.* Reconstitution after HCT is slowed by the use of immunosuppressive agents to prevent GvHD or by active GvHD itself. Infusions of prophylactic IV immunoglobulin for hypogammaglobulinemia are recommended for the first few months after transplant, and prophylactic oral antibiotics such as penicillin may be required for many months and sometimes years in patients with chronic GvHD due to the ever-present risk of serious infection. Patients also are at risk for *Pneumocystis jirovecii* pneumonia due to immunosuppression and cellular immunodeficiency and require prophylaxis therapy with trimethoprim-sulfamethoxazole, dapsone, or inhaled pentamidine.
6. *Transplantation-associated thrombotic microangiopathy (TA-TMA).* A number of stimuli including use of calcineurin inhibitors and sirolimus can trigger systemic injury to the vascular endothelium resulting in TA-TMA. This condition clinically may be similar to other entities such as thrombotic thrombocytopenic purpura (TTP), but the pathophysiology does not involve low levels of the enzyme ADAMTS-13 (the von Willebrand factor-cleaving protease) involved in blood clotting that degrades large vWf multimers, decreasing their activity. The mortality rates in affected patients may exceed 80%. There are several sets of specific diagnostic criteria. The approach is to eliminate agents such as calcineurin inhibitors and sirolimus, maintain adequate renal function, and consider blocking the complement system with eculizumab. Plasma exchange, potentially curative in TTP, has no proven efficacy in TA-TMA.
7. *Late effects.* Even survivors of HCT have a reduced life expectancy compared to age-matched controls in the general population. Malignancy can recur, and patients are at increased risk for second malignancy including other hematologic malignancies as well as solid tumors. The risk for the latter does not have a plateau.

Another potentially fatal but uncommon malignancy that can plague patients is termed *posttransplant lymphoproliferative disorder* (*PTLD*). It can arise after solid organ transplant or hematopoietic cell transplant. Impaired host immune

surveillance and Epstein-Barr virus (EBV)-infected B cells are key factors in the pathogenesis. When this condition develops, it usually is within the 1st year after transplant and reflects an unopposed proliferation of B cells. Subjects at highest risk are those who undergo a transplant using a graft that is devoid of T cells such as a T-cell-depleted graft or an umbilical cord blood graft or those who receive T-cell depletion in vivo. The B-cell hyperplasia may lead to further B-cell mutations and subsequently overt lymphoma. The initial strategy is to restore host T-cell immunity and provide anti-B-cell therapy with agents such as the monoclonal anti-B-cell antibody rituximab, but many patients require specific anti-lymphoma therapy.

Other late consequences include iron overload (due to numerous RBC transfusions), endocrine insufficiency (especially thyroid disorders), osteoporosis, and cataracts. In many ways, HCT can be viewed as an acceleration of the aging process.

Outcomes of Allogeneic Hematopoietic Cell Transplantation

Patient outcome is complex and depends upon patient-, disease-, and treatment-related factors including the underlying disease and stage at time of transplant, the previous therapy given, and the type of transplant (HLA-identical family member donor versus HLA-nonidentical family member donor versus HLA-identical unrelated donor). A few general observations:

1. The later in the disease course the HCT occurs, the lower the survival rate.
2. While in the past, unrelated donor transplant recipients generally did not fare as well as sibling-matched transplants because of increased incidence of GvHD and infections. With significantly better histocompatibility testing and donor, recipient matching, the outcomes for unrelated donor transplants now approach those of sibling-matched transplants.
3. Relapses still may occur, even years later, although the relapse rates are lower with allogeneic than with autologous HCT.
4. The transplant-related mortality rate at day 100 is approximately 15–20%, depending on how closely the donor and recipient are matched. The most frequent causes of death include relapse, GvHD, and infection.

Summary

HCT is a curative therapy for a number of hematologic disorders, both malignant and nonmalignant. Autologous HCT transplants have fewer immunologic complications but have higher rates of relapse after transplant. Allogeneic HCT have lower rates of relapse but have more immunologic complications, including GvHD, which can be fatal. Advances in HLA typing, supportive care, and newer immunosuppressive agents significantly have improved long-term survival rates after HCT over the past 10 years.

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Infections in Hematology Patients

38

Ola Blennow and Per Ljungman

Introduction

In hematology patients, the immunosuppression caused by the hematologic disease and its treatment usually predicts both the severity and type of infections. Neutropenia predisposes to severe bacterial infections and, if prolonged, for fungal infections, while an impaired T-cell function increases the risk for fungal and viral infections. Besides knowledge about the patients' immune status, the local resistance pattern, such as the frequencies of extended spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* and methillicin-resistant *Staphylococcus aureus* (MRSA), is important when deciding on empirical antimicrobial therapies. An important diagnostic principle in immunosuppressed patients is to "go for the bug" at the place of infection using invasive procedures, most often bronchoalveolar lavage (BAL) and biopsies.

Prevention of Infections

Prevention of infection in hematology patients consists of antimicrobial prophylaxis, prevention of exposure to infectious agents, and immune prophylaxis (vaccination; immune globulin).

Most studies of antibacterial prophylaxis have been performed with fluoroquinolones. Although the risk for neutropenic fever can be reduced, the effects on outcome have been variable, and its use is controversial due to the increased rates of resistance. If antibacterial prophylaxis is to be used, it should be reserved only to high-risk patients.

Antifungal and antiviral prophylaxis regimens are discussed in sections dealing with the specific pathogens further on in this chapter. Other preventive measures are described in Table 38.1.

Neutropenic Fever

A. Epidemiology:

1. Neutropenic fever is usually defined as a single temperature measurement of ≥38.3 °C (101 °F) or a temperature of ≥38.0 °C (100.4 °F) sustained over a 1-h period in a patient with ANC <500 cells/μL.
2. It is very common in patients with hematologic malignancies and occurs in more than 80% of patients with chemotherapy-associated neutropenia.
3. In approximately 50% of episodes, no diagnosis can be established (i.e., fever of unknown origin, FUO), whereas clinically documented infections and microbiological documented infections are diagnosed in around 25% of episodes each.
4. Bloodstream infections constitute the vast majority of microbiological documented infections. The epidemiology of bloodstream infections differs between centers, but generally there is a slight dominance of Gram-negative enteric bacteria (such as *E. coli*, *K. pneumoniae*, *P. aeruginosa*) over Gram-positive pathogens (such as coagulase-negative staphylococci, viridans streptococci, *E. faecium*). Antimicrobial resistance is rapidly increasing among both Gram-negative and Gram-positive isolates, most importantly extended ESBL production in *Enterobacteriaceae*.
5. The all-over mortality is low, around 5%, due to low mortality in patients with FUO. In patients with documented infections, the reported mortality

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Table 38.1 Prevention of infection in hematology patients

Measure	Patient group	Data source/study quality
Isolation procedures including hygienic measures	HSCT patients and other patients	Epidemiological data + knowledge about transmission routes
Food safety	HSCT and other patients	Good rationale for specific pathogens (listeria, salmonella). Otherwise unclear effects on the microbiome
Pets	HSCT and other patients	Good rationale, but limited data
Water safety	HSCT and other patients	Good rationale, but limited data (exception legionella and HAV)
Safe sex	HSCT and other patients	Some rationale (HSV) but no specific data
Antibacterial prophylaxis	HSCT and other patients	Several studies of mixed quality
Antifungal prophylaxis	HSCT patients, AML/MDS	Randomized studies
Antiviral prophylaxis	HSCT patients	Randomized studies against some viruses
	Other patients	Old studies of mixed quality
Immunoglobulin prophylaxis	HSCT patients	Old studies of mixed quality
	Other patients	Old studies of mixed quality
Vaccination	HSCT patients	Randomized studies of pneumococcal vaccination. Studies of mixed quality of influenza vaccination
		Studies of mixed quality of vaccination against other pathogens
	Other patients	Few controlled studies. Uncontrolled studies of mixed quality

HSCT allogeneic hematopoietic stem cell transplantation

rates are considerably higher: around 20% in patients with lung infiltrates, 35% in severe sepsis, and 50% in patients with septic shock [1].

B. Diagnostics:
   1. A careful clinical examination should be performed daily to identify possible infection focuses and detect clinical deterioration. Focuses that are easily overlooked include dental infections and perianal infections.
   2. Blood cultures should be taken through the central catheter and peripherally simultaneously, making it possible to identify catheter-related bloodstream infections (see section "CRBSI"). The total blood volume should be at least 40 ml (i.e., four bottles) to reach an acceptable sensitivity.
   3. Other cultures depend on the clinical signs of infection. Possible specimens include sputum, urine, skin, and stool samples (for *Clostridium difficile*).
   4. PCR for respiratory viruses should be performed in severely immunocompromised patients with respiratory symptoms especially in influenza season. PCR for other viruses is usually not part of the initial workup.
   5. In patients with respiratory symptoms, a thoracic CT scan should be performed. Chest X-ray is not meaningful in neutropenic patient because of low sensitivity and specificity (see also section "Pneumonia").
   6. BAL should be performed if the CT scan shows lung infiltrates (Table 38.2 and section "Pneumonia").
   7. New skin lesions/nodules should be biopsied and sent for microscopy and cultures, and fungal PCR should be considered.

C. Antimicrobial therapy:
   1. Empirical therapy:
      (a) *Neutropenic fever is a potentially life-threatening infection and treatment must always be initiated promptly. Infections with Gram-negative bacteria have been associated with high mortality, and the empirical therapy must therefore always include a broad Gram-negative coverage, including Pseudomonas species. Firsthand treatment options are presented in* Table 38.2 [3, 4].
      (b) *Vancomycin should not be added routinely to the empirical treatment because studies have clearly shown that this has no impact on mortality.*
      (c) *Oral antimicrobial treatment may be given from the start in selected low-risk patients* (Table 38.2) [5].
   2. Targeted therapy:
      (a) *Empirical therapy can be de-escalated to targeted therapy in microbiological documented infections, but not until the patient becomes afebrile. In neutropenic patients, fever is usually the only sign of a new infection and can be missed if antimicrobial coverage is narrowed down to a targeted treatment in a persistently febrile neutropenic patient* [2].
   3. Duration of therapy:
      (a) *Patients with FUO and still neutropenic: Until 48 h without fever.*
      (b) *Patients with FUO and non-neutropenic: Until resolution of fever.*
      (c) *Patients with microbiological and clinically documented infections: Same as for FUO, with the exception that the total duration of therapy should not be shorter than the normal treatment duration in non-neutropenic patients with the same diagnosis.*

Table 38.2 Empirical antimicrobial treatment of neutropenic fever [2]

Patient characteristics	Empirical firsthand treatment	Comment
No clinical focus, stable patient, no risk factor for resistant pathogen[a], MASCC score ≥21[b]	Consider outpatient treatment with ciprofloxacin + amoxicillin/clavulanate	Usually not applied to patients with, or anticipated, profound neutropenia for more than 7 days
No clinical focus, stable patient, no risk factor for resistant pathogen[a], MASCC score <21	Piperacillin/tazobactam, cefepime, ceftazidime	
MRSA colonization	Vancomycin should be added to the empirical treatment	
ESBL colonization (not carbapenem-resistant)	Imipenem/cilastatin, meropenem	
ESBL (carbapenem-resistant) or multidrug-resistant *Pseudomonas* colonization	Often combination treatment including colistin	Consider susceptibility data
Respiratory symptoms/focus	Piperacillin/tazobactam, cefepime	Avoid ceftazidime due to less activity against viridans streptococci
Abdominal symptoms/focus	Piperacillin/tazobactam, imipenem/cilastatin, meropenem	Avoid single treatment with cefepime and ceftazidime due to less anaerobic activity
Cellulitis (not associated with a central catheter)	Piperacillin/tazobactam, cefepime	Avoid ceftazidime due to less Gram-positive activity
Cellulitis around a central catheter	Vancomycin should be added to the empirical treatment	
Tunnel infection, port abscesses	Vancomycin should be added to the empirical treatment	Remove central catheter
Severe sepsis/septic shock	Imipenem/cilastatin or meropenem	
	Consider addition of amikacin and vancomycin	
	Add empiric antifungal treatment with an echinocandin if not on adequate antifungal prophylaxis	

MRSA methillicin-resistant *Staphylococcus aureus*, *ESBL* extended spectrum beta-lactamase

[a]Including prior colonization or infection with resistant pathogens, nosocomial infection, and prolonged hospital stay

[b]The Multinational Association for Supportive Care in Cancer (MASCC) risk index score: burden of febrile neutropenia (no or mild = 5, moderate = 3, severe = 0), systolic blood pressure >90 mmHg (5), no chronic obstructive pulmonary disease (4), solid tumor or hematologic malignancy with no previous fungal infection (4), no dehydration requiring iv fluid (3), outpatient status (3), age <60 years (2). Maximum = 26.

D. Neutropenic fever despite 72 h of treatment with broad-spectrum antibiotics:
   1. A management algorithm is presented in Fig. 38.1.
   2. If the new diagnostic workup is negative and the patient is stable, i.e., has not deteriorated, usually no change in therapy is necessary.
   3. If the neutropenic fever continues, a new cycle of diagnostic workup is initiated.
   4. Addition of empiric vancomycin to a stable patient has not been shown to decrease mortality and duration of fever and should be avoided.
   5. If the patient deteriorates, the antimicrobial therapy must be changed to include an even broader coverage. A carbapenem (meropenem or imipenem/cilastatin) should be prescribed if not already given. Empirical antifungal therapy should be initiated (see section "Empirical Antifungal Therapy"), and addition of amikacin and/or vancomycin considered.

Pneumonia

A. Epidemiology:
   (a) Respiratory symptoms and/or a new lung infiltrate are frequent in patients with hematologic diseases. In patients receiving treatment for acute leukemia, up to 30% may be affected with an associated mortality of over 20% [6].
   (b) The etiology often depends on the underlying immunosuppression (Table 38.3). In many cases, no etiology can be established.
   (c) Noninfectious reasons for a new infiltrate should be considered and include alveolar bleeding, heart failure, infiltration of the hematologic malignancy, organizing pneumonia, immune reconstitution syndrome, and damage from chemotherapy or radiation.

B. Diagnostics:
   (a) The clinical presentation is usually fever and/or respiratory symptoms such as cough, dyspnea, hemoptysis, and/or impaired oxygenation.

Table 38.3 Etiology of pneumonia

Patient characteristics	Bacteria	Virus	Fungus
Moderate immunosuppression[a]	*Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus*, Gram-negative enteric bacteria	Unusual	Pneumocystis pneumonia – low risk
T-cell suppression	As above +	Respiratory virus[b]	Pneumocystis pneumonia – high risk (if no prophylaxis)
	Mycobacterium tuberculosis	CMV	Invasive aspergillosis
		Adenovirus	Mucormycosis
			Other mold infections
Prolonged neutropenia (typically >10 days)	As above +	Unusual	Pneumocystis pneumonia – low to moderate risk (if no prophylaxis)
	Pseudomonas species		
	Nocardia species		
	Actinomyces species		Invasive aspergillosis
	Stenotrophomonas maltophilia		Mucormycosis
	Mycobacterium tuberculosis		Other mold infections

CMV cytomegalovirus
[a]Neutropenia <10 days, no treatment with high-dose glucocorticoids, no T-cell-directed therapy
[b]Depending on epidemiology. Includes influenza, parainfluenza 1–3, respiratory syncytial virus (RSV), enterovirus, coronavirus, rhinovirus, human metapneumovirus

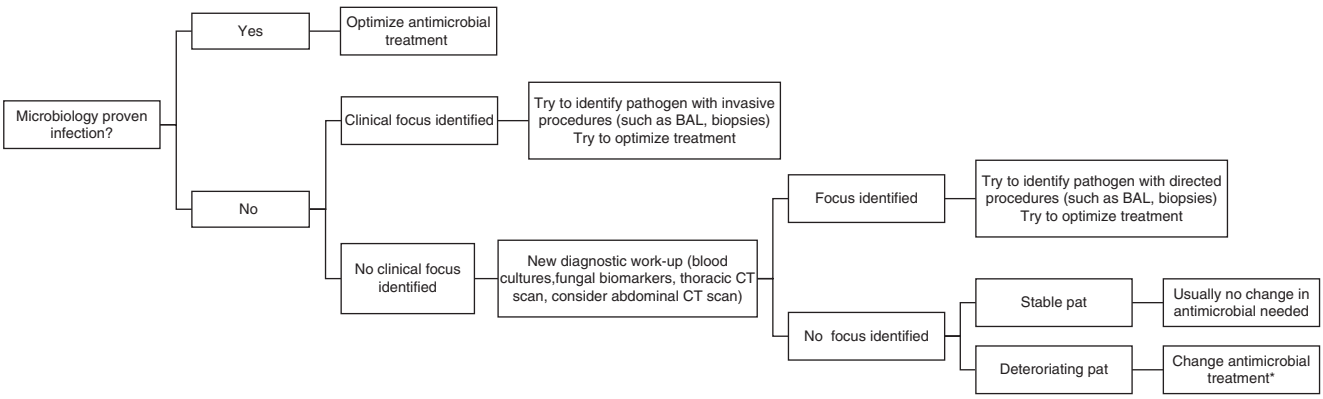

Fig. 38.1 Algorithm for management of prolonged neutropenic fever despite broad-spectrum antibiotics. *Change antibiotics to a carbapenem (meropenem or imipenem/cilastatin) if not already given. If receiving carbapenem consider adding amikacin, vancomycin, and an antifungal agent (see section "Empirical Antifungal Therapy")

(b) Findings on physical examination include tachypnea, impaired oxygenation, and lung crepitations.
(c) A thoracic CT scan should be performed early in patients with acute leukemia or T-cell suppression experiencing respiratory symptoms. Early detection of lung infiltrates indicating invasive mold disease or *Pneumocystis jirovecii* pneumonia (PCP), leading to early institution of antimicrobial treatment, has been shown to result in improved survival. Conventional chest X-ray has limited value in patients with hematologic diseases, especially when neutropenic, because of low sensitivity and specificity as compared to CT [6].
(d) The extent of microbiology sampling depends on the severity of the pneumonia and grade of immunosuppression (Tables 38.3 and 38.4).
(e) BAL is often required for establishing a microbiological diagnose and should be considered early in patients with acute leukemia or T-cell suppression with lung infiltrates (Tables 38.3 and 38.4). BAL should always be performed, unless contraindicated, if no response to initial empiric antimicrobial treatment.
(f) Coagulase-negative staphylococci, enterococci, and *Candida* species are generally not etiologically relevant for lung infiltrates even if found in BAL culture.

C. Treatment:
(a) Empirical treatment:
1. *In neutropenic patients and/or in nosocomial pneumonia, empirical treatment should include adequate Pseudomonas coverage besides cover-*

Table 38.4 Diagnostic tests in pneumonia and targeted treatment [2, 6]

Etiology	Diagnostics	First-line antimicrobial treatment	Other options	Comment
Bacteria				
Pseudomonas aeruginosa	Culture: sputum, BAL	Pip/taz, carbapenems, ceftazidime, cefepime	Colistin, ceftolozane/tazobactam	Consider combination treatment (aminoglycosides usually not effective in pneumonia)
Escherichia coli and *Klebsiella pneumoniae* with ESBL production	Culture: sputum, BAL	Carbapenems	Depends on resistance pattern	
Stenotrophomonas maltophilia	Culture: sputum, BAL	High-dose TMP/SMX	If TMP/SMX not possible: consider minocycline and moxifloxacin	In vitro susceptibility may not reliably predict clinical effect
Staphylococcus aureus, MSSA	Culture: sputum, BAL	Oxacillin		Vancomycin inferior
Staphylococcus aureus, MRSA	Culture: sputum, BAL	Vancomycin linezolid		Daptomycin should not be used in pneumonia due to high failure rate
Fungus				
Pneumocystis jirovecii (PCP)	PCR and IF: sputum, BAL	High-dose TMP/SMX	Primaquine + clindamycin pentamidine	Adjuvant treatment with high-dose glucocorticoids controversial
Aspergillus species	Culture and microscopy: sputum, BAL Biomarkers: serum, BAL[a] (PCR: BAL, biopsy)[b]	Voriconazole, isavuconazole	Lipid formulations of amphotericin B	Combination voriconazole and an echinocandin may be beneficiary in severe infections
Mucormycosis	Culture and microscopy: sputum, BAL (PCR: BAL, biopsy)[b]	Lipid formulations of amphotericin B	Isavuconazole	Posaconazole is also an option but less well documented
Fusarium species	Culture and microscopy: sputum, BAL (PCR: BAL, biopsy)[b]	Voriconazole lipid formulations of amphotericin B	Posaconazole	
Virus				
Influenza	PCR: nasopharyngeal swab, BAL	Oseltamivir		No controlled data
Respiratory syncytial virus (RSV)	PCR: nasopharyngeal swab, BAL	Ribavirin		No controlled data. Ribavirin likely reduces the risk of progression from upper to lower airway infection and the mortality in lower tract infection
Adenovirus	PCR	Cidofovir or brincidofovir		No controlled data
Cytomegalovirus (CMV)	PCR: BAL	Ganciclovir possibly with iv Ig	Foscarnet	No controlled data. The value of adding iv Ig is uncertain

Pip/taz piperacillin/tazobactam, *TMP/SMX* trimethoprim/sulfamethoxazole, *IF* immunofluorescence, *Iv* intravenous
[a]Galactomannan, beta-glucan, lateral-flow device (see section "Fungal Infections")
[b]May be considered (see section "Fungal Infections")

age of other Gram-negative enteric bacteria, S. pneumoniae, H. influenzae, and S. aureus. Suitable options include piperacillin/tazobactam, cefepime, meropenem, and imipenem/cilastatin [6].

2. *In non-neutropenic patients with community-acquired pneumonia, cefotaxime/ceftriaxone is a reasonable firsthand alternative if admitted to hospital, and amoxicillin/clavulanate or levofloxacin if treated as outpatients.*
3. *In patients with T-cell suppression, addition of trimethoprim/sulfamethoxazole (TMP/SMX) for empirical treatment of PCP should be considered if not on prophylaxis.*

(b) Targeted treatment:
   1. *Targeted therapy is outlined in* Table 38.4 (*and in the* sections "Fungal Infections" and "Viral Infections") [2, 6].

(c) Breakthrough infection or no response to empirical treatment:
   1. *A new thoracic CT scan should be performed to identify progression of infiltrates or new infiltrates compatible with invasive mold infection or*

PCP. It is also useful for deciding in which lobe(s) the BAL should be performed.
2. *BAL should always be performed if no contraindication* (Table 38.4).
3. *Change to meropenem/imipenem if receiving piperacillin/tazobactam, cefepime, or ceftazidime.*
4. *Consider addition of voriconazole as empirical treatment of invasive aspergillosis in patients not receiving mold-active prophylaxis and lipid formulation of amphotericin B for coverage of other mold infections in patients receiving mold prophylaxis.*
5. *Consider adding a fluoroquinolone if not already been prescribed for prophylaxis.*
6. *Consider adding high-dose TMP/SMX if there is risk of PCP and/or Stenotrophomonas maltophilia.*

Cather-Related Bloodstream Infection (CRBSI)

A. Epidemiology:
1. Different definitions of CRBSI exist, but common and clinical useful definitions are (1) growth in central blood culture at least 2 h before peripheral blood culture or (2) blood culture and catheter tip culture with growth of the same organism [7].
2. CRBSI is a well-recognized complication in hematologic patients with a reported incidence of up till 5.2 per 1000 catheter days. The causative organisms are most often coagulase-negative staphylococci, followed by *Staphylococcus aureus*, *Candida* species, and, more seldom, Gram-negative enteric bacteria, such as *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*.
3. Appropriate preventive measures are important to minimize CRBSI and include both education of the healthcare personnel handling the central venous catheter (CVC) and application of aseptic bundles during the insertion and use of the CVC. In addition, assessment of the necessity of the CVC should be made routinely with prompt removal when not needed [8].

B. Diagnostics:
1. Most often, the only clinical sign is fever, sometimes accompanied by signs of local infection at the insertion site or tunnel.
2. Blood cultures are simultaneously drawn peripherally and from the central catheter, preferably one set of blood cultures per lumen.
3. If the differential in time to positivity (DTP) is 2 h or more in favor of blood cultures drawn through the CVC, CRBSI definitions are fulfilled [7].
4. A diagnose of catheter-related infection can also be made without positive blood culture if there are undisputable signs of infection (erythema, swollenness, pain) over the catheter tunnel or port pocket.
5. In the case of positive central blood cultures with an organism known to cause CRBSI, negative peripheral blood cultures, and no local signs of infection, a catheter-related infection is suspected but not proven. A diagnostic algorithm in this scenario is outlined in Fig. 38.2.

C. Treatment: [7, 8]
1. Catheter removal, together with targeted antimicrobial therapy, remains the mainstay of treatment of CRBSI. Removal should always be performed in septic, hypotensive patients, in patients with complicated CRBSI such as tunnel and port infections, and in CRBSI caused by *Candida* species.
2. Vancomycin should be added in septic, hypotensive patients with suspected CRBSI and/or local signs of infection such as tunnel infection, port abscesses, or cellulitis around insertion site.
3. In CBRSI caused by *Pseudomonas aeruginosa* and *Staphylococcus aureus*, removal of catheter is associated with lower relapse rates and better outcomes and should be performed routinely.
4. In CBRSI caused by other Gram negatives than *Pseudomonas*, antibiotic-lock therapy can be tried if removal of the catheter is not feasible.
5. In CRBS with coagulase-negative staphylococci and no local signs of infection, the catheter can often be retained with the use of antibiotic-lock therapy.
6. Antibiotic-lock therapy involves installing a high concentration of an antibiotic to which the causative microbe is susceptible in the catheter lumen. The solution should be left in the lumen without interruption for at least 8 h per 24 h, but longer if possible. When using a catheter retaining strategy, 14 days of combined systemic therapy and antibiotic-lock therapy are usually recommended. When blood cultures become negative, iv therapy may be switched to oral.
7. If the catheter is removed and there are no signs of local or metastatic infection, treatment duration is usually 7 days, except for *Staphylococcus aureus* (14 days) and *Candida* species (14 days after first negative blood culture).

Typhlitis (Neutropenic Enterocolitis)

A. Typhlitis is a potentially serious complication in neutropenic patients characterized by fever, abdominal pain, and thickening of the cecum and adjacent ileum [9].

B. The pathogenesis involves intestinal damage associated with neutropenia and mucositis, followed by microbial invasion with inflammation and ulceration.

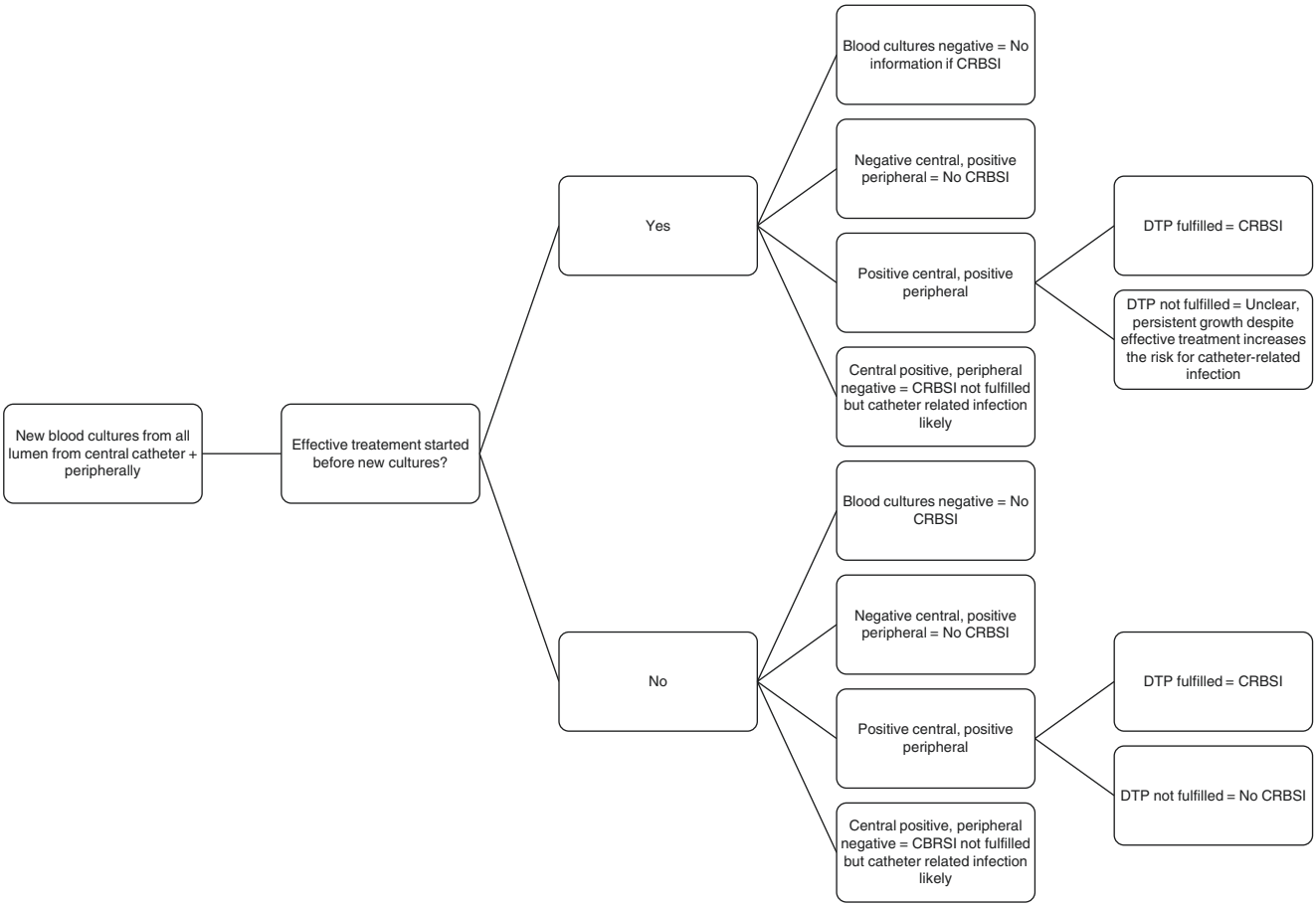

Fig. 38.2 Diagnostic algorithm for CRBSI. DTP differential in time to positivity, CRBSI catheter-related bloodstream infections

C. The reported incidence varies, depending on the definitions and intensity of chemotherapy, from 5% in all patients with hematologic malignancies up to 28% in patients with AML [9].
D. Ultrasound or CT scan showing bowel wall thickening in a neutropenic patient with fever and abdominal pain is diagnostic.
E. Blood cultures and *Clostridium difficile* assays should be performed, and antimicrobial treatment covering Gram-negatives, including *Pseudomonas aeruginosa*, and anaerobes should be instituted promptly. Suitable options include monotherapy with meropenem, imipenem/cilastatin, or piperacillin/tazobactam or a combination of cefepime and metronidazole.
F. Translocation of *Candida* species may occur, and empirical treatment/prophylaxis with a candida-active agent, such as fluconazole or an echinocandin, should be considered.
G. Bowel rest and G-CSF to shorten the duration of neutropenia may be considered, but evidence for effect are lacking.
H. Surgery should be avoided unless absolute necessary (perforation or massive bleeding) [9].

Fungal Infections

A. Empirical and preemptive antifungal therapy:
   1. Epidemiology:
      (a) *Prolonged neutropenic fever despite broad-spectrum antibiotic therapy is common, and empirical antifungal therapy based on this indication only will lead to administration of antifungals to around one third of neutropenic patients. However, the majority of these patients do not have an* invasive fungal disease *(IFD), and indiscriminate empirical antifungal therapy will thus expose patients to unnecessary and potentially harmful drugs.*
      (b) *The incidence of IFD is approximately 5–10% in high-risk patients receiving candida-active prophylaxis, such as AML/MDS patients receiving remission-intended chemotherapy. In patients receiving mold-active prophylaxis, usually posaconazole or voriconazole, the reported frequency of breakthrough IFD is considerably lower, between 0.5% and 1.5%* [10].

(c) *Patients anticipated to have a short period of neutropenia (<10 days) have a low risk of IFD, especially if receiving candida-active prophylaxis.*

2. Diagnostics:
   (a) *Initial diagnostic workup includes blood cultures, a thoracic CT scan, and biomarkers in blood or serum (usually galactomannan (GM) and/or (1 → 3)-β-D-glucan (beta-glucan); see* sections "Invasive Aspergillosis" and "Invasive Candida Infections") [11].
   (b) *Many commercial blood culture systems have a good sensitivity for Candida species if no concomitant bacteremia, with the possible exception of Candida glabrata. Thus, if the suspicion of invasive Candida infection is high, or if concomitant bacteremia is suspected, blood cultures with better sensitivity for yeast ("mycosis bottles") should be included.*
   (c) *In case of pulmonary infiltrates, a BAL should be performed* (Table 38.4) [12].
   (d) *If no candida-active prophylaxis has been administered, CT scan of the abdomen, looking for signs of hepatosplenic candidiasis, may be considered.*
   (e) *New skin lesion/nodules should be biopsied and sent for microscopy, cultures, and PCR.*
3. Treatment:
   (a) *First-line options include lipid formulations of amphotericin B, an echinocandin, or voriconazole.*
   (b) *The best choice of treatment in patients with suspected breakthrough infection while on mold-active treatment has not been evaluated in clinical trials, but a switch to another drug class is recommended. In patients receiving mold-active azoles, change to a lipid formulation of amphotericin B is a logical choice as this gives a broad coverage against other mold infections than aspergillosis.*
   (c) *The timing of empirical antifungal treatment depends on choice of strategy.*
      1. In the empirical approach, antifungal treatment is initiated after 72–96 h of neutropenic fever despite broad-spectrum antibiotics, even if there are no other signs or findings suggestive of IFD. As discussed above this approach will expose many patients to unnecessary and potentially harmful treatment and is not recommended.
      2. In the preemptive approach, patients are screened in blood/plasma two to three times per week with one or more biomarkers, most often GM. If a biomarker becomes positive, a thoracic CT scan is performed, and if infiltrates are present, BAL are performed, and empirical antifungal therapy started. In patients with negative CT scans, screening with biomarkers continues, and no antifungals are started. If a biomarker becomes positive again, the procedure with a thoracic CT scan is repeated. Studies have shown that this approach significantly reduces the use of antifungals without reducing overall survival, although the frequency of diagnosed IFD was slightly higher than in the empirical arm [13].
      3. In the diagnostic approach, biomarkers and thoracic CT scans are performed in high-risk patients with neutropenic fever for more than 3–5 days despite broad-spectrum antibiotics. If infiltrates are found, a BAL should be performed. If both biomarkers and CT scan are negative and the patient is stable, empirical antifungal therapy can be withheld.
   (d) *In high-risk neutropenic patients deteriorating despite broad-spectrum antibiotics, empirical antifungal treatment should be initiated even if the diagnostic work-up for IFD is negative.*
   (e) *The duration of empirical treatment should be as short as possible, and no longer than until resolution of neutropenia, if no IFD has been diagnosed.*

B. Invasive *Candida* infections:
1. Epidemiology:
   (a) *Candidemia in neutropenic patients is a life-threatening infection, which can lead to acute disseminated candidiasis, a sepsis-like syndrome, and death* [14].
   (b) *Chronic disseminated candidiasis (hepatosplenic candidiasis) can occur as a complication of candidemia in neutropenic patients.*
   (c) *Important risk factors are increased colonization of the gastrointestinal tract and other mucosal surfaces by Candida species, disruption of the protective mucosal barrier due to chemotherapy, and decreased phagocytic capacity due to neutropenia.*
   (d) *The incidence depends on the depth and duration of neutropenia, on the degree of mucosal disruption, and whether prophylaxis has been administered.*
   (e) *For recommendations of prophylaxis, please see* Table 38.5.
2. Diagnostics:
   (a) *Most often the only symptom in candidemia is fever. Right upper quadrant discomfort, fever, nausea, and elevation of liver enzymes after reso-*

Table 38.5 Recommended anti-fungal prophylaxis [10]

Risk classification	Clinical examples	Type of prophylaxis	First-line agent	Alternative agents
High risk	Acute leukemia or myelodysplasia with remission intended treatment,	Anti-mold and anti-candida	Posaconazole (Fluconazole)[c]	1. Voriconazole
	Severe GVHD[a]			2. Liposomal AmB, itraconazole, echinocandins, aerosolized liposomal AmB (+ fluconazole)
	Extensive chronic GVHD			
	Extensive T-cell directed therapy[b]			
Low risk	Autologous HSCT	Anti-candida	Fluconazole	Echinocandins
	Allogeneic HSCT without GVHD			Itraconazole
	Intensive/dose-escalating therapy for lymphoma			
Very low risk	Standard therapy for lymphoma	None		
	Chronic myeloid leukemia			
	Other myeloproliferative neoplasms			

GVHD graft-versus-host disease, *AmB* amphotericin B
[a]Steroid dependent or refractory or grade III–IV
[b]Such as T-cell-directed antibodies (thymoglobulin, alemtuzumab) and/or prolonged treatment with high-dose corticosteroids
[c]Only if low incidence of mold infections. May also be given as part of an integrated care strategy together with a mold-directed diagnostic approach (includes weekly screening with biomarkers during periods of high risk)

lution of neutropenia are the most common findings in chronic disseminated candidiasis.

(b) *Cultures of blood, and other samples collected under sterile conditions, are the diagnostic gold standard for invasive candidiasis and should always be performed. However, since cultures are hampered by low sensitivity (overall sensitivity for blood cultures is estimated to be around 50%), several other diagnostic tests have been developed* [14].
   1. (1 → 3)-β-D-Glucan (beta-glucan) is a cell wall constituent of *Candida* species, *Aspergillus* species, *Pneumocystis jirovecii*, and several other fungi. Beta-glucan detection in plasma may detect cases of invasive candidiasis days to weeks prior to positive blood cultures and shorten the time to initiation of antifungal therapy. However, a positive result should be interpreted with caution because of a low positive predictive value due to poor specificity. Moreover, true-positive results are not specific for invasive candidiasis, but rather suggest the possibility of an invasive fungal infection.
   2. Candida PCR in blood has been shown to be helpful but comparisons have been hampered by lack of standardization. The FDA approved a commercial *Candida* PCR in 2014, but clinical data is limited.

(c) *In hepatosplenic candidiasis contrast-enhanced CT, MRI, PET-CT, and ultrasound can all be used for identification of microabscesses in the liver and spleen.*

(d) *Abscesses and metastatic embolus to the skin should be biopsied and sent for microscopy, culture, and PCR.*

3. Treatment:
   (a) *Recommendation of antifungal treatment and duration is outlined in* Table 38.6 [14, 15].

C. Invasive Aspergillosis (IA):
1. Epidemiology:
   (a) *Aspergillus species and other filamentous fungi are ubiquitous in the environment. Inhalation of fungal spores is the most common portal of entry and sinopulmonary disease the most frequent clinical manifestation. Dissemination can occur through hematogenous spread* [16].
   (b) *The most important risk factors are prolonged neutropenia and T-cell impairment.*
   (c) *Patients at high risk include those receiving induction therapies for AML/MDS, especially in those with refractory or relapsed acute leukemia.*
   (d) *Patients undergoing allogeneic hematopoietic cell transplant (HCT) have an increased risk of IA, with the most important risk factors being the severity and duration of graft-versus-host disease* [17].
   (e) *Prevention includes reducing exposure to fungal spores (such as hospital rooms with high-efficiency particular air (HEPA) filtration, avoiding gardening and construction sites) and administration of mold-active prophylaxis to patients at high risk* (Table 38.5).
2. Diagnostics:
   (a) *The diagnosis of IA is based on the combination of radiology and microbiological findings in a susceptible host and is often challenging to reach.*
   (b) *The clinical presentation is usually fever with or without cough, hemoptysis, and pleural pain.*

Table 38.6 Treatment of invasive *Candida* infections [14]

	Candidemia, non-neutropenia	Candidemia, neutropenia	Hepatosplenic candidiasis
Treatment			
Initial	1. An echinocandin	1. An echinocandin	1. Lipid formulation of AmB (3–5 mg/kg) or an echinocandin
	2. Fluconazole (only if stable and no azole prophylaxis)	2. Lipid formulation AmB (3–5 mg/kg)	
Step-down	Fluconazole in clinically stable patients with susceptible isolates	Fluconazole (or voriconazole if mold coverage is wanted) can be used during persistent neutropenia in clinically stable patients with susceptible isolates	After several weeks of AmB or an echinocandin treatment may be changed to fluconazole in patients who are unlikely to have a fluconazole-resistant isolate
Duration	14 days after first negative blood culture if no metastatic complication	Until resolution of neutropenia, but not shorter than 14 days after first negative blood culture if no metastatic complication	Until lesions resolve on repeat imaging, usually several months
Ophthalmological examination	Yes, within the 1st week after diagnosis	Yes, within the 1st week after resolution of neutropenia	Yes, as part of the investigation
CVC removal	Most often but individualized decision, as early as possible if CVC is thought be the source	More seldom as the dominating source is the gastrointestinal tract	Individualized decision

AmB amphotericin B, *CVC* central venous catheter

(c) *A thoracic CT scan should always be performed. Typical findings include nodules, consolidative lesions, wedge-shaped infarcts, and the halo sign: a nodule >1 cm in diameter surrounded by a ground-glass opacity reflecting hemorrhage* [16, 18].

(d) *Diagnostic BAL should always be performed unless contraindicated* (Table 38.4)*, and sputum should be sent for microscopy and culture* [12, 16].

(e) *Since the diagnostic yield of microscopy and culture is limited, other tests have been developed. All the available tests have weaknesses and best results have been reported when combining two (or more) tests* [11, 19].

1. Galactomannan (GM) antigen test is the most used. GM constitutes part of the *Aspergillus* fungal wall and can be detected in serum and BAL (and in CSF) with a commercial test that is FDA approved. In neutropenic hematology patients, the sensitivity is around 70% when tested in serum and higher in BAL. In patients receiving mold-active treatment, the sensitivity is significantly reduced.
2. Aspergillus-specific lateral-flow device is an antigen test with a performance similar to GM with the advantage of providing a test result within minutes but is not as well documented [19].
3. Beta-glucan (see also section "Invasive *Candida* Infections") has a sensitivity similar or just below the one of GM but poor specificity due to high rate of false-positive results and because a true-positive test is not specific for *Aspergillus* species. A commercial test is available and has FDA approval.
4. PCR in blood and BAL have been shown to have a diagnostic performance comparable to GM, and combined negative GM and PCR tests in BAL performed in a patient not receiving mold-active treatment have a high negative predictive value. However, until recently few of the PCR assays had been standardized and validated, and since no assay has been approved by the FDA, it is difficult to recommend PCR for routine use as of yet [20, 21].

3. Treatment:

(a) *First-line treatment is voriconazole or isavuconazole, with isavuconazole generally being better tolerated. Therapeutic drug monitoring is generally recommended when using mold-active azoles to make sure that therapeutic levels are reached* [15, 16, 22].

(b) *Alternative treatment is lipid formulations of amphotericin B*[16].

(c) *Primary therapy with a combination of voriconazole and an echinocandin may be considered in patients with severe disease, especially if profound and prolonged neutropenia.*

(d) *The clinical course depends on the duration and severity of neutropenia. The size of the lesions usually increases during the 1st week of treatment and then remains stable for another week. Repetition of CT scan is not recommended before 2 weeks after the start of treatment unless clinical deterioration is seen.*

(e) *Treatment duration is minimum 6–12 weeks but depends on the underlying disease and immunodeficiency. Patients who require subsequent immunosuppression should receive secondary prophylaxis to prevent recurrence* [15, 16].

(f) *Adjunctive measures should be considered: Ongoing immunosuppression should be minimized or eliminated altogether if possible, colony-stimulating factors may be considered in neutropenic patients, and granulocyte transfusion may be considered in refractory infections.*

D. Other mold infections of importance:

1. Epidemiology:

(a) *Other mold infections than IA are seen in around 15% of all invasive mold infections, either alone or in combination with IA* [22].

(b) *Mucormycosis (formerly known as zygomycosis) is caused by members of the order Mucorales and is the dominating etiology to non-IA mold infections. Mucormycosis is very aggressive with reported mortality rates between 24% and 49%.*

(c) *Fusariosis is caused by an invasive infection with Fusarium species and is the second most common non-IA mold infection. The outcome is usually poor, and largely dependent on the recovery of the immune status of the host, particularly neutropenia* [23].

(d) *Risk factors for non-IA mold infections are the same as for IA,* i.e., *prolonged neutropenia and T-cell suppression.*

2. Diagnostics:

(a) *The most common manifestations of mucormycosis are rhino-cerebral and pulmonary infections. Diagnostics rely on radiology in combination with microscopy and culture of biopsies and BAL. PCR may be considered, but no commercial test approved by FDA exists. Typical findings on CT scan are the same as for IA, except that the reversed halo sign, an area of ground-glass opacity surrounded by a ring of consolidation, is more frequent in mucormycosis and thus suggestive of the disease. Negative GM and aspergillus PCR in a BAL performed on suspicion of mold infection is also suggestive of non-IA infections such as mucormycosis.*

(b) *Fusariosis in hematology patients is most often disseminated, and pulmonary lesions are found in almost 50% of cases. The radiologic picture is similar to IA. Nodular and papular skin lesions (which should be biopsied) are common and blood cultures are often positive. Fusarium species interact with the GM test so that the test may become positive even in the absence of concomitant IA* [23].

3. Treatment:

(a) *Mucormycosis is a very aggressive infection in hematology patients and treatment must be initiated promptly. First-line agent is high-dose liposomal amphotericin B combined with surgery. Isavuconazole has recently been evaluated (retrospectively) and was found to have an efficacy similar to that of liposomal amphotericin B and is an option if liposomal amphotericin B cannot be used. Surgery should be performed if possible* [15, 17, 24].

(b) *The drug of choice for the treatment of invasive fusariosis is either voriconazole or liposomal amphotericin B* [23].

E. *Pneumocystis jirovecii* pneumonia (PCP):

1. Epidemiology:

(a) *Transmission of Pneumocystis jirovecii occurs during the first years of life* via *person-to-person contacts, usually asymptomatically or as mild infection of the upper respiratory tract.*

(b) *P. jirovecii pneumonia (PCP or PJP) carries a high mortality in hematology patients and early recognition and treatment are critical for successful outcome* [25].

(c) *PCP can occur both from previous colonization and from new person-to-person transmission.*

(d) *Prophylaxis should be administered to patients at risk such as acute lymphoblastic leukemia (ALL), allogeneic HCT, >4 weeks of treatment with corticosteroids (≥20 mg/day prednisone), treatment with alemtuzumab, and treatment with fludarabine/cyclophosphamide/rituximab. Prophylaxis should also be considered in patients with other risk factors, such as treatment for lymphoma* [25, 26].

(e) *First-line prophylaxis is TMP/SMX. Second-line choices include aerosolized pentamidine, dapsone, and atovaquone* [26].

2. Diagnostics [27]:

(a) *Clinical presentation is most often fever, nonproductive cough, dyspnea, and/or impaired oxygenation.*

(b) *Chest X-ray has low sensitivity. Thoracic CT scan usually shows bilateral, patchy ground-glass opacities, predominantly in the perihilar regions. However, the radiology findings are non-specific, so to establish the diagnosis of PCP, the pathogen must be identified.*

(c) *Identification of P. jirovecii can be difficult because hematology patients generally have a lower fungal load than non-hematology patients have.*

(d) *BAL is the preferred specimen since a gradient of fungal load is expected with the highest load in*

BAL fluid and the lowest in upper respiratory sample. In addition, BAL can identify other pathogens, which is important since coinfection is common in patients with PCP. If BAL cannot be performed, sputum, preferably induced sputum, is preferred over other upper respiratory specimens such as oral washing, nasopharyngeal swabs, or nasal swabs.

(e) *P. jirovecii cannot be cultivated, and identification is based on PCR and microscopy using immunofluorescence (IF) techniques.*

(f) *PCR has higher sensitivity than IF, but a positive result may be difficult to reliably discriminate between colonization and infection.*

(g) *A positive IF corresponds to diagnose of PCP regardless of specimen.*

(h) *PCP is ruled out if PCR is negative in BAL, whereas a negative PCR test in sputum or other upper respiratory samples does not rule out infection.*

(i) *The combination of a positive PCR and negative IF cannot distinguish between colonization and infection.*

(j) *A negative test for serum beta-glucan has a high negative predictive value.*

3. Treatment [28]:

(a) *Treatment should be instituted promptly to optimize the chance of successful outcome.*

(b) *First-line treatment is high-dose iv TMP/SMX for ≥14 days, followed by prophylaxis until immunosuppression is resolved.*

(c) *Second-line treatments include oral primaquine + iv or oral clindamycin, iv pentamidine, and oral atovaquone.*

(d) *Administration of glucocorticoids cannot be generally recommended and must be based on case-by-case basis.*

Viral Infections

A. Epidemiology

Viral infections can broadly be divided into latent/persistent viruses that after primary infection remain in the patient for many years/for life and viruses that are only present for a short period (days–weeks). Viral infections are particularly important in the most immunocompromised especially allogeneic HCT. Many different viruses can cause the same clinical syndromes (Table 38.7), *and therefore specific diagnostic procedures are required to allow correct management.*

B. Diagnosis

1. Detection of antibodies

Detection of antibodies as evidence of infection is a standard technique in healthy immune competent individuals with IgM as a sign of recent infection and IgG as evidence of past infections. In hematology patients, serology is rarely useful for diagnosis of ongoing infection but is very important in determining risks for viral infections occurring posttransplantation.

2. Detection of viruses or viral components

Traditional virus isolation in cell culture is rarely performed today. Instead methods detecting viral antigens or nucleic acids (nucleic acid testing – NAT) have become the standard for virus identification. NAT can also be used to determine viral load and through mutation analysis/sequencing detect resistance against antiviral drugs.

3. Diagnosis on tissue

Table 38.7 Clinical syndromes and possible viral pathogens (incomplete list)

Pneumonia	Encephalitis	Hepatitis	GI disease
CMV	HHV-6	CMV	CMV
Influenza	Adenoviruses	EBV	HSV
Adenoviruses	HSV	Adenoviruses	Adenoviruses
RSV	VZV	HBV	EBV
Parainfluenza	CMV	HCV	VZV
Metapneumovirus	Measles	VZV	Rotaviruses
Coronaviruses	JCV	HAV	Noroviruses
Rhinoviruses	EBV	HEV	Astroviruses
Measles	Rabies		
VZV	West Nile virus		
Bocavirus			

GI disease gastrointestinal disease, *CMV* cytomegalovirus, *HHV-6* human herpes virus 6, *EBV* Epstein-Barr virus, *HSV* herpes simplex virus, *RSV* respiratory syncytial virus, *VZV* varicella zoster virus, *HBV* hepatitis B virus, *HCV* hepatitis C virus, *JCV* JC virus, *HAV* hepatitis A virus, *HEV* hepatitis E virus

To diagnose end-organ disease, virus should be detected in the affected organ preferably by specific staining of tissue.

C. Herpesviruses

Herpesviruses are latent/persistent viruses and frequently reactivate in immunosuppressed patients.

Herpes simplex virus (HSV)-1 and HSV-2:

(a) *The rates of seropositivity in the population increase with age. HSV-1 usually causes localized infections in the orofacial area but can occur in other locations. HSV-2 usually causes localized infections in the urogenital tract. HSV-1 encephalitis is the most severe manifestation but is rare in hematology patients as in the normal population. HSV-2 can cause meningitis.*

(b) *NAT is the diagnostic method of choice for both viruses.*

(c) *Prophylaxis against HSV-1 and HSV-2 with acyclovir/valaciclovir is recommended to all seropositive patients undergoing HSCT and should be considered for other intensively treated hematology patients. These drugs are also first choice for treatment* [29].

(d) *Resistance to acyclovir can develop and occurs in approximately 5–15% of patients depending on risk profile. Foscarnet is the first-line treatment for acyclovir-resistant virus.*

D. Cytomegalovirus (CMV):

(e) *The rate of CMV seropositivity increases with age and varies with geographical location. CMV seropositivity is a risk factor for non-relapse mortality and decreased survival after allogeneic HCT. A CMV-seronegative donor should, if available, be used for a CMV-seronegative patient* [30–32].

(f) *Approximately 60% of CMV-seropositive patients develop active CMV infection after HSCT, most commonly during the first 2 months, while the risk for a CMV-seronegative patient receiving a graft from a CMV-seropositive donor is approximately 30%. For other hematology patients and patients undergoing autologous HSCT, the risks are much lower. Patients receiving anti-T-cell therapy are at an increased risk.*

(g) *Most CMV infections are asymptomatic or cause low-grade fever possibly with depressed bone marrow function. End-organ CMV disease occurs in 2–10% of HSCT patients, most commonly gastrointestinal disease. CMV pneumonia is still associated with a high mortality. Other end-organ manifestations are retinitis, hepatitis, and encephalitis. Risk factors are allogeneic HCT, acute GVHD, and intensive T-cell suppression. CMV end-organ disease in other groups of hematology patients is uncommon* [30].

(h) *Weekly blood monitoring of allogeneic HCT patients is recommended using either quantitative PCR or the pp65 antigenemia assay. No fixed cutoff can be recommended since it depends on the risk profile of the patient and the exact assay used, but many centers use cutoffs around 1000 IU/ml* [33].

(i) *Monitoring is not routinely recommended for autologous HCT patients or other hematology patients. There is no information regarding cutoffs for therapy in these types of patients.*

(j) *The diagnosis of CMV disease requires symptoms and/or signs + CMV detected from the involved organ. CMV in blood combined with symptoms from an organ is not enough for making the diagnosis CMV disease* [30, 34].

(k) *Possible strategies for management are prophylaxis, preemptive therapy, and treatment of end-organ disease.*

(l) *Letermovir has been shown to be effective as prophylaxis reducing the risk for clinically significant CMV infection and can also reduce all-cause mortality* [35].

(m) *Preemptive therapy based on detection of CMV by monitoring is an effective strategy in high-risk patients such as allogeneic HCT recipients. Ganciclovir and valganciclovir are first-line drugs for treatment of CMV infection and disease. High-dose iv immunoglobulin (Ig) has been used in combination with ganciclovir or treatment of CMV pneumonia, but its usefulness has not been proven in studies* [33].

(n) *Antiviral resistance develops in 0–10% of patients depending on their risk profile. Second-line drugs are foscarnet and cidofovir. The main limitation with these drugs is side effects. Case reports and small case series exist with the use of leflunomide or artesunate for resistant and refractory CMV.*

(o) *CMV specific T-cells are an option if available.*

(p) *New antivirals are currently undergoing clinical development (maribavir, letermovir, brincidofovir).*

E. Varicella-zoster virus (VZV):

(a) *VZV causes to different diseases: primary VZV infection causes chickenpox and reactivation of VZV causes herpes zoster (HZ, shingles).*

(b) *In countries not using general vaccination of children, most individuals have experienced chickenpox in childhood.*

(c) *Chickenpox can become severe in immunocompromised individuals.*

(d) *The risk for HZ increases with age and is also increased in immunocompromised individuals.*

(e) *Most HZ cases are self-limiting and give local symptoms. However, disseminated infections mimicking primary varicella and visceral cases sometimes without skin rash occur with poor outcome unless rapidly diagnosed and treated.*

(f) *Most cases can be easily recognized by the characteristic rash. PCR from the vesicular rash is the diagnostic method of choice, but immunofluorescence might also be used. PCR on blood is useful in the diagnosis of visceral cases.*

(g) *Acyclovir and valaciclovir (valacyclovir) are the drugs of choice for prevention and treatment of VZV infections. Prophylaxis is recommended in patients at high risk for HZ such as HSCT recipients and myeloma patients treated with proteasome inhibitors. IV acyclovir should be given to patients with primary varicella and to disseminated and visceral HZ cases* [36].

(h) *Management of severe local HZ such as zoster ophthalmicus and zoster oticus needs close collaboration with appropriate specialists.*

F. Epstein-Barr virus (EBV):

(a) *The rate of seropositivity increases with age and most adults are seropositive. EBV constantly replicates in seropositive individuals without causing symptoms.*

(b) *End-organ EBV disease including meningitis, hepatitis, and pneumonia occurs but is rare also in severe immunocompromised individuals.*

(c) *The most important complication to EBV is post-transplant lymphoproliferative disease (PTLD) occurring in high-risk allogeneic HCT recipients.*

(d) *EBV can also be a trigger of hemophagocytic lymphohistiocytosis (HLH) in patients with hematologic malignancies.*

(e) *In high-risk allogeneic HCT patients, monitoring in blood with quantitative PCR is indicated. There is no established cutoff since there is no standardized test* [37].

(f) *Symptoms and signs of PTLD are unspecific. Increased LDH, lymphadenopathy, and fever are common. CT scan for detection of splenomegaly and/or intrathoracic or intra-abdominal lymphadenopathy should be considered. Biopsy of lymph nodes is needed to prove the diagnosis.*

(g) *There is no specific antiviral therapy effective against EBV.*

(h) *Rituximab is the preferred intervention both for pre-emptive therapy against EBV PTLD based on increasing EBV viral load and for treatment of established PTLD* [37].

(i) *Reduction of immunosuppression should be attempted if possible* [37].

(j) *EBV-specific T-cells are an option if available.*

G. Other herpesviruses

Human herpesviruses (HHV)-6 A and B are the most common cause of viral encephalitis after allogeneic HCT. Their importance in other hematology patients is less well defined. The diagnosis is made by MRI + PCR on cerebrospinal fluid. Available antiviral drugs for treatment are foscarnet and ganciclovir/valganciclovir [38].

H. Community-acquired respiratory viruses:

(a) *Respiratory viruses circulate in the community, and the risk for patients with hematological diseases to become infected reflects the local epidemiology. Respiratory syncytial virus (RSV), influenza viruses, and adenoviruses are best known to be able to cause disease in hematology patients, but reports of severe disease have been also reported with many other respiratory viruses including parainfluenza viruses, metapneumoviruses, and rhinoviruses. Nosocomial spread occurs which is why infection control is paramount. Most infections are mild and self-limiting, but severe infections can occur especially in allogeneic HCT recipients and patients receiving intensive immunosuppressive therapy* [39–42].

(b) *The diagnosis is in most centers based on multiplex PCR on respiratory samples either from the upper airways (nasal, nasopharyngeal, or throat samples) or from the lower respiratory tract (BAL fluid). Most existing assays can detect 10–15 different respiratory viruses. Other techniques can be used such as antigen tests and immunofluorescence but have limitations most important that they detect only one virus.*

(c) *The most important part of management is prevention including avoiding infected individuals, handwashing, and influenza vaccination of patients, family members, and staff. It should be recognized that these infections can be spread by individuals having very limited symptoms.*

(d) *If an allogeneic HCT candidate presents before start of conditioning with symptomatic infection with a respiratory virus, postponing the transplant should be considered.*

(e) *Antiviral therapy has a limited role in most of these patients but can be considered in the most severely immunocompromised patients such as allogeneic HCT patients.*

(f) *Therapeutic options with data supporting efficacy, although there are no controlled studies, are ribavirin for RSV, neuraminidase inhibitors for influenza, and cidofovir/brincidofovir for adenovirus infections.*

I. Hepatitis viruses

Several different viruses can cause liver disease in hematology patients with the most important being hepatitis B

and C. These viruses are significant pathogens in patients with hematological diseases and screening should always be performed. Increasing evidence implicates hepatitis E virus (HEV) as an important pathogen and that this virus can be transmitted through blood products [43].

1. Hepatitis B virus (HBV):
   (a) *HBV infection is widely distributed in the world with varying prevalence in different populations. Chronic infections are associated with liver cirrhosis and the development of hepatocellular cancer.*
   (b) *Immunosuppressive therapy is associated with HBV reactivation in chronically infected individuals. HBV reactivation can result in severe liver disease including liver failure.*
   (c) *Reactivation is more common in HBsAg-positive individuals but also HBsAg-negative individuals; anti-HBc-positive individuals receiving intensive immunosuppression such as after HSCT or anti-B-cell antibodies can reactivate HBV.*
   (d) *Patients with high risk for reactivation should receive antiviral prophylaxis against HBV.*
2. Hepatitis C virus (HCV):
   (a) *HCV infection is widely distributed in the world with varying prevalence in different populations. HCV infection is associated with an increased risk for non-Hodgkin lymphoma, and chronic HCV infection is associated with liver cirrhosis and hepatocellular cancer.*
   (b) *Chronically infected patients with HCV can have flare-ups of liver disease during immunosuppressive therapy, and liver monitoring is therefore indicated.*
   (c) *Antiviral therapy for HCV infection is rapidly evolving, and expert advice regarding treatment options should therefore be obtained.*

J. Other viruses

Many different viruses can cause symptomatic infections in hematology patients. These include the polyomaviruses (JC and BK) [44], *viruses causing gastroenteritis outbreaks (norovirus, rotavirus), and viruses spread through mosquito bites (yellow fever, dengue, Zika, chikungunya). The knowledge about the clinical importance of these viruses is limited, but it is likely that severe disease can develop in the most severely immunocompromised patients.*

Summary

Early diagnosis and specific treatment are key factors in reducing the morbidity and mortality of infections in hematology and HCT patients. The rise of multiresistant organisms is a major threat and infection control is therefore of uttermost importance.

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Correction to: Concise Guide to Hematology

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Correction to:
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This book was inadvertently published with incorrect edition information in Preface as "The third edition" whereas it should be "The second edition".

This book was inadvertently published with following errors in Chapter 5:

In Figure 5.1, the amounts of iron transferred daily from the Spleen and bone marrow macrophages (RES) to Plasma transferrin and from Plasma transferrin to the Bone marrow-Erythropoiesis were incorrectly shown as 20 gm. This should instead be 20 mg. We regret the error.

In page 34, at the top of the left column, the sentence is incorrect. The sentence should read, 'However, in patients with iron deficiency, ferritin usually does not rise above 200 μg/L (ng/mL) even if inflammation is present.' We regret the error.

In page 34, in the left column in the second to last line of list (E), there is a typo, "Inflmmation" – it should read "Inflammation".

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