16 Pathology of Lymphoreticular Tissues

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16.1 Introduction and Scope of This Chapter

Hematopathology is a discipline in which the routine methods of clinical and morphologic analysis are interwoven with routine laboratory as well various ancillary techniques for diagnosis and management of hematolymphoid lesions. Such lesions broadly may be divided into non-neoplastic (like viral, tubercular, autoimmune, drug induced) and neoplastic. Neoplastic lesions involving nodes could be hematolymphoid or non-hematolymphoid neoplasms. Scope of this chapter is hematolymphoid neoplasms (HLN) as defined by the WHO 2017 Classification of HLN and others [1–3]. Here we discuss the morphology of normal lymphoreticular tissues (primarily lymph nodes, bone marrow trephine biopsy, spleen, and thymus) followed by an approach to diagnosis and subtyping of HLN based on morphology and various ancillary techniques, primarily immunohistochemistry (IHC) (Figs. 16.1, 16.2, 16.3, and 16.4). Bone marrow aspirate is not a part of the scope of this chapter.

Diagnostic hematopathology has been a nemesis for pathologists. Contributing factors to the difficulty of diagnosing HLN include lack of trained hematopathologist and lack of widespread use of various ancillary techniques. To be an expert in hematopathology, a pathologist needs to see an adequate number of cases of such lesions on a daily basis along with various ancillary techniques. It is desirable to have a trained hematopathologist who has expertise in both his-

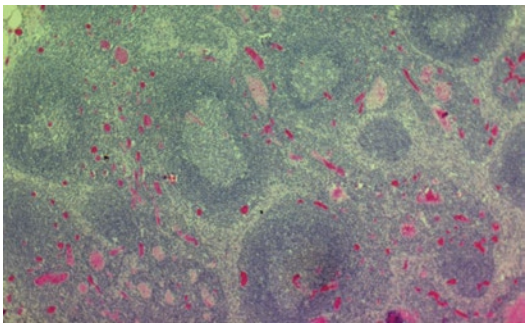

Fig. 16.1 Normal LN showing secondary follicles with outer marginal zone, middle mantle zone, and inner germinal center

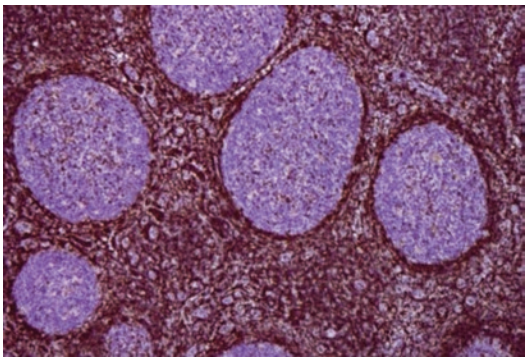

Fig. 16.2 Normal LN showing bcl2 stain—IHC

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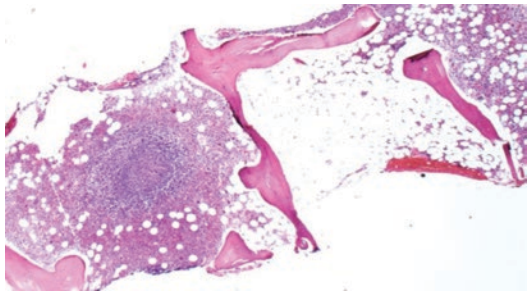

Fig. 16.3 BM biopsy showing secondary follicles in intertrabecular location

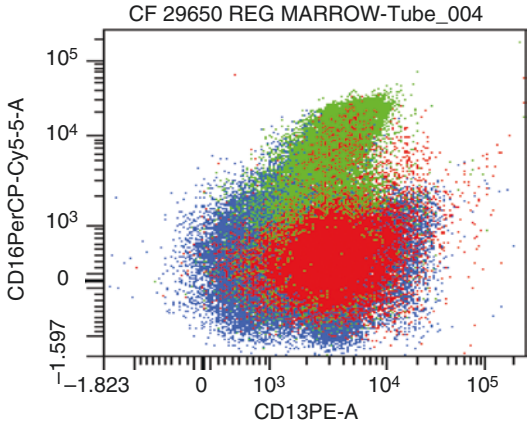

Fig. 16.4 Multicolor immunophenotyping of bone marrow aspirate showing myeloid maturation patterns

topathology (LN, extranodal lymphoid lesions, BM biopsy) and liquid hematology (peripheral blood smear and BM aspirate) with training in various ancillary techniques. Such kind of training programs are available in the USA where they have fellowship programs in hematopathology which encompasses both liquid and solid hematolymphoid tissues with an exposure in various ancillary techniques. In India, most of the hematopathology training programs (post residency fellowship or a DM program) are tilted toward liquid hematology (peripheral blood and bone marrow) and a very little exposure to solid tissue hematopathology (including lymph nodes). Similarly, body fluids may be seen and reported in another division (cytopathology) in most centers. Though IHC is available in many centers, flow cytometry, cytogenetics, and molecular diagnostics are available in few selected centers. Very few residency programs in India encompass exposure in all these areas including various ancillary techniques.

HLN range from the most indolent to highly aggressive neoplasms. They arise from cells of immune system at different stages of differentiation. Various hematolymphoid tissues include peripheral blood, bone marrow, lymph nodes, thymus, spleen, Waldeyer's ring, and lymphoid tissue at mucosal and other sites. Some of these HLN present as leukemia (primary involvement of peripheral blood and bone marrow), some as lymphomas (primarily involving lymph nodes, spleen, thymus, and other solid organs), and some as combined. Clinical course can change over the course of the disease. Therefore, the terms leukemia and lymphoma may be used interchangeably. HLN constitute approximately 8–10% of all neoplasms in our practice at Tata Memorial Hospital, Mumbai with equal contribution from both solid and liquid HLN. Recent years have brought an explosion of new diagnostic tools to the pathology of HLN, which have permitted more precise disease definition and recognition of factors that can predict prognosis and response to treatment with targeted therapies. Thus, it is imperative for a hematopathologist to have an updated clinical knowledge and also various modalities to reach a final impression. Standard protocols should be followed for various procedures, and it starts with sampling, grossing, processing of tissues so as to finally obtain a good-quality well-stained thin H&E section.

16.2 Brief About HLN

Older classification systems of lymphoid and myeloid neoplasm were separate; however, these have come under one umbrella, latest one being the WHO 2017 Classification of HLN. It has been broadly divided into tumors of precursor lymphoid neoplasms, mature B-cell neoplasms, mature T- and NK-cell neoplasms, Hodgkin lymphoma, immunodeficiency-associated LPD, histiocytic and dendritic cell neoplasms, and myeloid neoplasms, each having further subtypes (Table 16.1).

Table 16.1 Broad subtypes of hematolymphoid tumors [1]

Classification of hematolymphoid neoplasm	Subclassification
Myeloid neoplasms	(a) Myeloproliferative neoplasms (b) Mastocytosis (c) Myeloid/lymphoid neoplasm with eosinophilia and gene rearrangement abnormalities of PDGFRA, PDGFRB, FGFR1, or PCM1-JAK2 (d) Myelodysplastic/myeloproliferative neoplasms (e) Myelodysplastic syndromes (f) Myeloid neoplasms with germline predisposition (g) AML and related precursor neoplasms (h) Blastic plasmacytoid dendritic cell neoplasm (i) Acute leukemia of ambiguous lineage
Lymphoid neoplasms	(a) Precursor lymphoid neoplasms (B, T, and NK lymphoblastic lymphoma/leukemia) (b) Mature B-cell neoplasms (c) Mature T- and NK-cell neoplasms
Hodgkin lymphoma	(a) Nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) (b) Classical HL • Nodular sclerosis • Mixed cellularity • Lymphocyte rich • Lymphocyte depleted
Immunodeficiency-associated lymphoproliferative disorders (LD)	(a) LD with primary immune disorder (b) Lymphoma associated with HIV infection (c) Post-transplant lymphoproliferative disorders (d) Other iatrogenic immunodeficiency-associated LD
Histiocytic and dendritic cell neoplasms	(a) Histiocytic sarcoma (b) Tumors derived from Langerhans cells (c) Indeterminate dendritic cell tumor (d) Interdigitating dendritic cell sarcoma (e) Follicular dendritic cell sarcoma (f) Fibroblastic reticular cell tumor (g) Disseminated juvenile xanthogranuloma (h) Erdheim–Chester disease

Lymphoid neoplasms can occur anywhere as lymphoid cells exist all over. Nodal lymphomas include lymph node, Waldeyer's ring, bone marrow aspirate and biopsy, thymus, and spleen. Remaining may be considered as extranodal. In the following sections, we discuss HLN as per primary organ involved. There is a huge list of lymphoma subtypes; hence, to simplify, we may study it under various headings such as low-grade and high-grade types, pediatric and adult types, and nodal and extranodal types. Common HLN subtypes in adults include diffuse large B-cell lymphoma, chronic myeloid leukemia (CML), chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), acute myeloid leukemia (AML), Hodgkin lymphoma (HL), follicular lymphoma (FL), T-cell lymphoblastic lymphoma (T-LL), anaplastic large cell lymphoma (ALCL), Burkitt lymphoma (BL), etc. (as seen in our practice). Common HLN subtypes in children include precursor B-cell lymphoblastic lymphoma/leukemia (B-LL), T-LL, HL, ALCL, and BL. Kindly refer WHO 2017 Classification of HLN for its detailed classification for myeloid neoplasms and WHO-EORTC 2005 classification for cutaneous lymphomas [1, 4, 5].

16.3 Investigations Required

Workup of a case of HLN needs multimodality approach. Routine investigations include complete blood counts, serum proteins (globulin and albumin), erythrocyte sedimentation rate (ESR),

serum uric acid, serum calcium, creatinine, lactate dehydrogenase (LDH), beta 2 microglobulin, etc. Morphological evaluation of the tissue like peripheral blood smear, bone marrow aspirate/biopsy, lymph node and extranodal biopsy specimen (including spleen) is of utmost importance. Various ancillary techniques can detect proteins [immunophenotyping by immunohistochemistry (IHC) and flow cytometric immunophenotyping (FCI)], messenger RNA (in situ hybridization), or changes in DNA [Southern blot, PCR, fluorescence in situ hybridization (FISH), and gene expression profile]. Next-generation sequencing (NGS) has joined the long list of the investigative tools in workup of hematolymphoid neoplasms (still at very few centers in west). Ann Arbor staging system is used to stage these lymphomas [6, 7]. Thus, common diagnostic modalities include morphology, cytochemistry (e.g., myeloperoxidase, nonspecific esterase, Perls' Prussian blue), immunophenotyping (IHC and FCM), cytogenetics (both conventional cytogenetics and FISH), and molecular diagnostics (including NGS). Consent for all laboratory procedures as well as investigations are essential for diagnosis, starting from fine needle aspirate to DNA/RNA studies, and it should be a part of the initial consultation with the patient. In all cases, adequate clinical information is essential to assess the risk of the specimen and plan the investigations accordingly. Request forms must include relevant clinical as well as laboratory information including complete blood counts, biochemical investigations, and results of any preceding investigations such as peripheral blood FCI. Findings of radiological investigations like ultrasound abdomen, CT scan, and PET/CT scan are useful.

16.4 Lymph Node: Interpretation and Lymphomas

16.4.1 Introduction and Indication for Biopsy

In adults, under normal conditions, only the inguinal nodes are palpable as 0.5–2.0 cm nodules. Similarly, in children, small 0.5–1.0 cm cervical nodes may be palpable. It may be difficult to define absolute indications for LN biopsy as tuberculosis is one of the commonest causes of lymphadenopathy, which can be diagnosed on FNAC with special stains for mycobacteria and other microorganisms (AFB, GMS, PAS) and culture. FNAC may be a screening test for any lump, and it will help decide if a biopsy is required. However, any lymphadenopathy (solitary or generalized) which is of size >1.5 cm, long-standing (4–6 weeks), firm to hard, movable, non-tender, and suggestive of lymphoma/neoplasm must be biopsied. It is advisable to do a whole node excision biopsy of the largest palpable node. Other indications may include persistent lymphadenopathy, mediastinal mass, abdominal mass or any other extranodal mass, and unexplained fever. History of persistent B symptoms, hepatosplenomegaly, etc. further indicates the need of a biopsy of any palpable mass. Frozen section diagnosis is avoided as it might lead to loss of tissue and also cause freezing artifacts, thereby limiting morphological interpretations with paraffin sections. Needle core biopsy (NCB) is getting popular these days, and role of FNAC is diminishing in many areas. It is done primarily when the lesion is in inaccessible sites (retroperitoneal, mediastinal) or else in incapacitated sick patients. But number of NCB has increased in past, and we get to see larger number of core biopsies even from palpable nodes where a whole node biopsy would have given much more information.

Histopathological examination is the primary mode of diagnosis for any patient with a clinical diagnosis of lymphoma. However, before invasive procedures are attempted, a complete blood count with a manual differential count should be performed as the presence of cytopenia or increased leukocyte counts may suggest bone marrow involvement. In such cases, peripheral blood smear with FCI may provide a lead and also the diagnosis in many cases. FCI has its limitations, as it does not provide morphology (architecture and cytology) of the lesion, and also difficult to diagnose lesions like Hodgkin lymphoma (owing to number and

nature of tumor cells). FCI provides invaluable information of background cells. In addition, many lymphomas are easily diagnosed based on FCI like hepatosplenic gamma-delta T-cell lymphoma, hairy cell leukemia, etc. FCI may obviate the need for invasive tests and risks of anesthesia in a significant number of cases. In patients who present with acute severe respiratory distress due to airway obstruction (superior vena cava syndrome due to a mediastinal mass), every attempt should be made to make a diagnosis before starting steroids or chemotherapy; otherwise, it may be difficult to diagnose. A diagnostic tap of pleural fluid or ascites may reveal tumor cells in T-lymphoblastic and Burkitt lymphoma, respectively (similarly precursor B-cell lymphoblastic lymphoma in cerebrospinal fluid). Laparotomy and resection of the bowel may be indicated in patients who present with intussusception or intestinal obstruction, the cause being a lymphomatous process.

Few patients being referred to tertiary care centers for further management might have already received some preliminary treatment in the form of steroids, heavy metals, local herbs, or blood transfusion. This might cause a temporary decrease in tumor load leading to a delayed diagnosis. In such situations, a trephine biopsy done upfront at the primary center is crucial, as this paraffin block may be used to perform IHC to further classify the acute leukemia. Thus, it is suggested that a BM biopsy can be done upfront in all cases of HLN along with BM aspirate, especially in acute leukemias. Such paraffin blocks are invaluable material, as IHC can be performed at a later stage even if there are no blasts seen on peripheral blood or repeat BM aspirate. BM biopsy is also useful in evaluating metastatic deposits, fibrosis, granulomas, etc. Bone marrow aspirates in partially treated acute leukemias may help in detecting minimal residual disease (MRD) in acute leukemias, extremely important in country like ours where most patients get steroids before they reach a tertiary care cancer center. MRD detection may help in picking up scanty cancer cells and hence a definitive diagnosis for further management.

16.4.2 Procedure, Collection, and Transport of Lymph Node Biopsy Material

Lymph node biopsy should be performed by a surgeon (likewise endoscopic biopsy by a gastroenterologist and skin biopsy by a dermatologist). A pediatric surgeon should preferably perform LN biopsy in a child. Whole node biopsy of the largest palpable node should be done. (do not cut the lymph node and send it to two or more laboratories at one time). NCB may be done for non-palpable lesions (inaccessible sites). Multiple cores may be obtained from deep-seated lesions such as abdominal, mediastinal, and retroperitoneal nodes. Tissue should be handled gently to avoid crushing artifacts. Do not use blunt needles or forceps, so as to avoid crushing of nodal tissue. Many surgeons practice NCB even on the large-sized palpable nodes for want of time and ease, and this should be discouraged unless otherwise indicated. Whole node biopsy of the largest palpable node is the mantra. If lymph node biopsy cannot be transported to the histopathology laboratory immediately, lymph node should be sliced serially (3–4 mm thin) perpendicular to its long axis and fixed in optimum quantity of buffered formalin (10 times the volume of the tissue biopsied). This will ensure optimal preservation of morphology and good results on IHC. In hospital-based laboratories, LN may be immediately submitted fresh to the laboratory for grossing and other ancillary tests. Specimens should reach the laboratory immediately after collection since cytogenetic analysis requires live cells for culture and RNA degrades rapidly. This requires close liaison with the operating theater staff because lymph node biopsies are frequently done by junior surgeons/trainees and are added to the end of operation theater list and thus reach laboratory late in the day. In cases where a specialized test like FCI or cytogenetic analysis is to be carried out at a remote location, transport or appropriate tissue culture media should be used. A part of the sample may be sent for snap freezing for the preservation of sample for ancillary molecular investigations. If the clinical impression is of an infective lesion, a fresh sample should be sent

directly to the microbiology laboratory for culture and sensitivity testing. For histopathology, the sample should be immersed in buffered formalin for 24–48 h for adequate fixation. Mention the number of lymph nodes and the size of the largest node. The lymph node may be sectioned perpendicular to the long axis of the node. This orientation provides the greatest assessment of the architecture. Majority of the tissue is put in formalin for histopathology laboratory. The bisected LN tissue should be fixed in formalin for 24–48 h; less than this might lead to poor preservation of cytological detail and can make the tissue difficult to interpret. Standardization of fixation makes IHC more reliable. Prolonged fixation makes IHC more difficult and recovery of DNA from paraffin blocks unreliable. In case of a small lymph node and needle-core biopsies, there may be material just enough for histopathology processing, and no additional investigations may be possible. However, if the specimen received is a whole fresh lymph node, which has sufficient volume, the specimen can be divided. Major chunk of the tissue specimen is sent for histological sections (in formalin or other fixative) and a part may be divided for DNA/RNA or FCI studies. Most labs in India prepare paraffin blocks only, which is still the gold standard for lymphoma diagnosis. Flow cytometry immunophenotyping has picked up in the last decade and so.

16.4.3 Histopathology and Ancillary Techniques: Lymph Node and Extranodal Tissues

Morphology: LN (and extranodal) biopsy sections should always be stained with Hematoxylin and Eosin. Scanner view (2×) is the most important, followed by low-power examination. Scanner reveals extranodal fat, capsule, and above all architectural patterns within the node. Abnormal patterns, infiltrates, and then cytological interpretation are picked up in the scanner view (using less light and lowering the condenser). *IHC* is required in almost all suspected cases of HLN for diagnosis and further subtyping. Cases like classical HL may be diagnosed based on morphology; however, IHC is required in most cases to exclude a wide range of morphological differential diagnoses from viral infections to a high-grade B- or T-cell lymphoma. IHC may be performed by manual or automated methods. *For FCM*, fresh lymph node samples are best analyzed as early as possible, preferably within 6–8 h. If sample has to be transported to a reference laboratory, a laboratory should have standard operating procedures (SOPs) for disaggregating and fixation of the sample in a transport media. *For cytogenetics*, a fresh sample of the specimen (BM aspirate and lymph node) that you want to specify in tissue culture medium should be sent for cytogenetic analysis. Cytogenetic analysis should be performed only once the morphological impression is made. This will help in saving costs in unnecessary screening. FISH may be done on imprint smears as well as on tissue sections. If fresh samples are processed for metaphases, these should be stored, and analysis is attempted after morphological assessment of the sample indicates a need for cytogenetics. The cytogenetic laboratory may store cell suspensions. EDTA may be used as an anticoagulant for molecular tests such as RT-PCR. A sample of fresh tissue may be rapidly frozen and stored for subsequent analysis if required.

16.4.4 Normal Histology and Immunophenotypic Profile

Normal lymph nodes are small, about 1 cm in size, but the size can vary depending upon the site and activity. It is important to understand normal histology along with IHC patterns of normal lymph nodes biopsied from various sites to understand different compartments and dynamic nature of the lymph node. Though it is a dynamic structure and no two normal nodes look similar; broadly, a lymph node may be divided into different compartments mainly capsule, cortex, paracortex, sinuses, and medulla. The understanding of physiology including normal architectural and functional compartment of lymph node is essential to make a diagnosis of pathology of

node including nodal lymphoma. Lymph node is a dynamic structure where lot of immunological responses occur to various stimuli like bacterial, viral, or parasitic infections, autoimmune disorders, drug-induced reactions, etc. The lymph node reacts to each of these stimuli differently, and accordingly its histology varies. There may be slight variation in its histology depending upon anatomical site of node. For example, the thoracic nodes show anthracotic pigment, inguinal and pelvic nodes show de novo sclerosis, mesenteric nodes have significant sinus histiocytosis, and spleen and its nodes may show a prominent marginal zone. Multiple reactive (non-metastatic) axillary nodes dissected in a case of mastectomy might show different morphological patterns (dynamic nature of the nodes). Varying morphological features on a scanner view in different nodes may include follicular hyperplasia, prominent T zone with features of dermatopathic lymphadenitis, and sinus histiocytosis, and an occasional node might show distended medullary cords rich in plasma cells. Various nodules can be seen in a low-power examination of the node. These may be B-cell nodules (common) or T-cell nodules. Among B nodules, it might be a primary follicle or a secondary follicle (has a germinal center). Section from an edge of a secondary follicle will appear as a nodule without germinal center (like section from an edge of a boiled egg).

These nodules (rich in mature looking small lymphoid cells) from an edge of a secondary follicle may mimic a primary B follicle or a low-grade B-cell lymphoma. The lymphoid cells in these nodules will diffusely express CD20 and bcl2 (with a low MIb1 and hardly any CD23) and may be diagnosed as low-grade B-cell lymphoma/marginal zone lymphoma on tiny/needle core biopsies of abdominal/retroperitoneal nodes. Bcl2 is a tricky stain. It is positive in most lymphoid cells in a normal node including marginal and mantle zone cells. It is negative primarily in centrocytes and centroblasts of the secondary follicle. CD4+ T follicular helper cells within germinal center will also express bcl2, and excess of these bcl2+ T cells may lead to misdiagnosis of follicular lymphomas in some situations. Sinuses are common sites of deposits of carcinomas, ALCL, etc. Paracortical T zone is rich in small lymphoid cells in a background of high endothelial capillaries. It may show a mottling pattern rich in interdigitating reticulum cells, which may weakly express cytokeratin called as cytokeratin-positive interdigitating reticulum cells (CIRCs). Pigment-laden macrophages are found here in cases of dermatopathic lymphadenitis (with a prominent T zone). Such conditions and also viral infections may lead to an increase in large-sized mononuclear cells (immunoblasts) in the T zone and may mimic RS cells. Immunoblasts express CD20 and Pax5 (strongly) and CD30; however, classic RS cells (morphology is important) express CD30, CD15, and EBV and weakly express Pax5 (generally CD20−). Nodes with granuloma are classically seen in infective conditions, and common microorganisms must be excluded starting from *Mycobacterium tuberculosis* (caseating granulomatous inflammation). Special stains like AFB, GMS, and PAS for microorganisms must be performed in such cases. Granulomas may also be seen in toxoplasmosis and in lymphomas like HL and Lennert's T-cell lymphoma. Nodes with patches of necrosis with increased apoptosis (karyorrhexis) may be seen in Kikuchi Fujimoto disease (mimic high-grade lymphoma). Here crescentic histiocytes within the necrotic patches express AMPO and plasmacytoid dendritic cells express CD123.

16.4.5 Immunophenotypic Profile of Normal Reactive Lymph Node

Normal lymph nodes reveal a spectrum of histological features. Immunophenotyping of lymphomas as B- or T-cell type is not straightforward as many of the lymphomas exhibit a polymorphous population, and majority of the cells in the background may be non-neoplastic, as seen in T-cell histiocyte-rich B-cell lymphoma (TCHRBCL), nodular lymphocyte predominant HL (NLPHL), and cHL (tumor cells are very scanty). High-grade lymphomas like angioimmunoblastic T-cell lymphomas may mimic cHL, TCHRBCL, and even a reactive node.

Pathologist has to decide on which are the cells of interest (tumor cells) because they may be small size and innocuous looking (like PTCL-NOS) or else may be scanty in nature (like HL, TCHRBCL, etc). Small size of lymphoblastic lymphomas may mimic small size of small lymphocytic lymphoma (SLL) cells. Thus, identifying cells of interest is the key. Morphology followed by extensive IHC panel for T, B, NK cells will help. Deciding on cells of interest may depend on morphology and sometimes on IHCs. Similarly, interpretations of IHC stains may be tricky and not be straightforward. CD5 expression in tumor cells in mantle cell lymphoma may be weaker than the background CD5/CD3-positive T cells (strongly positive). Similarly, Pax5 expression in RS cells in cHL is weak in comparison with brightly lit reactive B cells in the background. Internal control is as important as is external control (Table 16.2). Plasma cells and immunoblasts are the internal control for CD30, while histiocytes and eosinophils are for CD15. CD34, and cyclinD1 may highlight endothelial cells. CD10 and bcl6 will light up the germinal centers. MIB-1 is almost 100% seen within the germinal centers of secondary follicles, where bcl2 is negative. BCL2 may highlight scanty CD4+ T helper cells within the germinal center. Similarly, CD21, CD23, and CD35 are good markers to highlight compact germinal centers, where they stain follicular dendritic cells.

Table 16.2 IHC expression pattern of normal lymph nodal cells

Germinal center (GC) cells: CD10+, BCL6+, and BCL2−
Germinal center B cells: CD20+, CD79a+, Pax5
Germinal center T cells: CD3+, CD4+, CD57+, PD-1 (programmed death-1)+
Follicular dendritic cells: CD21+, CD23+, CD35+
Proliferation index (Ki67 or MIB-1 labeling index): high in GC (almost 100%)
Mantle zone cells: CD20+, CD79a+, CD5+, IgD+, IgM+, BCL2+
Marginal zone cells: CD20+, CD79a+, IgM+, IgD−/+, BCL2+
Paracortical T cells: CD3+, CD4+ or CD8+, CD43+, CD7, CD5, CD2
Histiocytes: CD68+, CD163, CD43+, S100−, CD1a− (S100 and CD1a will be positive in Langerhans cells)
Plasma cells: CD138+, CD38+, CD19+, Ig+, kappa or lambda light chains, EMA+/−, CD20−

16.4.6 Lymphoma Diagnosis

Lymphoma can have a wide range of clinical, morphological, and immunological findings. In our practice, the commonest subtypes of lymphoma are DLBCL and HL. Diagnosis of lymphoma requires multidisciplinary approach. Regardless of the type of lymphoma, initial evaluation of patient should include careful history and physical examination. Important clinical information includes age and sex of the patient, duration of symptoms, presence of B symptoms (like fever, weight loss, night sweats), lymphadenopathy, hepatosplenomegaly, and skin lesions. The baseline radiological investigations (CT scan, PET scan) help in staging and follow-up of the disease response evaluation. The biochemical parameters like albumin and globulin levels, ß2 microglobulin levels, serology for HIV, HBV, HCV, and hematological parameters like hemoglobin, platelet count, and lymphocyte percentage (absolute) are important baseline investigations. All these investigations are complementary to each other. All these parameters help to confirm the diagnosis and identify those manifestations of the lymphoma that might require prompt attention and also aid in the selection of further ancillary investigations, if required, for optimal characterization of lymphoma and to allow the best choice of therapy. Though it is a combined diagnostic approach, for convenience sake the approach to lymphoma diagnosis has been divided into four parts:

1. Morphological/histopathological approach
2. Immunophenotypic analysis
3. Cytogenetic studies
4. Molecular studies

16.4.6.1 Morphological/ Histopathological Approach to Lymphoma Diagnosis

Optimal fixation and processing of lymph node is important for morphological as well as immunohistochemical interpretation. A good quality H&E section is a key to lymphoma diagnosis. It is important to have a low-power lens in the microscope, preferably 1× (most expensive) or else a 2× lens.

For a pathologist, once the H&E-stained slide of suspected hematolymphoid lesion is on the microscope, the dilemma of lymphoma verses non-lymphoma starts. Common non-neoplastic lymphoid proliferations at these sites may be further subdivided into various categories based on etiology and morphological patterns. To take an example of a benign nodal lesion, we may have the following common patterns:

1. Follicular/nodular patterns are commonly seen in reactive follicular hyperplasia, viral infections, autoimmune disorders like rheumatoid arthritis lymphadenitis, hyaline vascular Castleman disease, progressive transformation of germinal centers, mantle and marginal zone hyperplasia.
2. Predominantly sinusoidal pattern is seen in reactive sinus histiocytosis, Rosai Dorfman disease, and hemophagocytic syndrome.
3. Paracortical T-zone expansion may be seen in dermatopathic lymphadenitis, granulomatous lymphadenitis (tubercular and others), Kimura's disease, toxoplasmosis, systemic lupus erythematosus, Kikuchi lymphadenitis, Kawasaki disease, and inflammatory pseudo tumor (IgG4-related diseases).
4. Diffuse patterns are commonly seen in viral infections such as infectious mononucleosis, cytomegalovirus infection, herpes simplex lymphadenitis, and also because of drugs.

The above benign lesions may mimic a lymphoma. Though morphology remains the cornerstone in diagnostic approach, a detailed history with various ancillary techniques including clonality studies may be required to reach a final diagnosis.

First question is whether we are dealing with a lymphoma or a reactive/infective/autoimmune disorder leading to lymphadenopathy. Many drug-related adenopathies (phenytoin, isoniazid, iodides, tetracycline, sulfonamides, allopurinol, etc.) may give rise to an abnormal pattern mimicking lymphoma. Dilantin-associated lymphadenopathy may mimic a high-grade lymphoma, and drugs like thiomercazol may lead to increase in hematogones like cells in the bone marrow, thus mimicking acute leukemia. Clinical history, along with ancillary techniques like IHCs and FCI (flow cytometric immunophenotyping), helps confirm reactive lymphoid cell patterns including hematogones. Many viral infections like EBV- and HIV-associated adenopathy may mimic cHL, T-cell histiocyte-rich B-cell lymphoma and PTCL-NOS, plasmablastic lymphomas, etc. Tubercular nodes on histology may resemble Hodgkin lymphoma or Lennert's lymphoma, a variant of peripheral T-cell lymphoma. Many patients diagnosed and treated as tuberculosis on the basis of fine needle aspirate might have a hematolymphoid neoplasm. This must be confirmed on a biopsy examination. Kikuchi Fujimoto disease (necrotizing lymphadenitis) with abundant karyorrhexis and plasmacytoid dendritic cells (which express CD123) may resemble high-grade NHL. Such cases require a thorough workup to exclude a possibility of an autoimmune disease and tuberculosis. Thus, a detailed history, thorough examination, complete autoimmune workup, and also special stains for fungus and mycobacteria may be mandatory in such situations. Castleman disease may mimic plasmacytoma and cHL. Light chain clonality and markers like CD15 and CD30 might help differentiate. All these are made complicated sometimes by IHC stains; to give an example, CD30+ B-immunoblasts are seen scattered all over in EBV adenopathy. They also express EBVLMP1 by IHC or else EBER by ISH. These may be confused as RS cells of cHL. However, these are generally mononuclear cells in morphology and also express LCA, CD20, and Pax5 strongly while may be negative for CD15. Thus, IHC alone will not help in differentiating these benign lymphoid proliferations from neoplastic ones. It is important to rule out the possibility of related benign entities and obtain adequate history. Toxoplasmosis commonly occurs in posterior cervical and occipital nodes (with a classical triad of follicular hyperplasia, microgranulomas, and monocytoid B-cell hyperplasia). A large-sized axillary or inguinal node of long duration in an otherwise asymptomatic patient suggests a Nodular Lymphocyte Predominant Hodgkin lymphoma (NLPHL). Patent sinuses, mottled T

zone, and preserved secondary follicles favor a benign node and, however, may be preserved in many lymphomas like AITL. Nodal architecture is well preserved in ALCL and metastatic carcinomas where scanty tumor cells might be seen in sub-capsular sinuses.

The second question is whether it is a hematolymphoid neoplasm or some other tumor. Common differential diagnoses include round cell tumors, carcinoma, melanoma, germ cell tumors, etc. depending upon the architecture and cytology of the tumor cells. Large cell lymphomas (like DLBCL, ALCL) may mimic deposits of carcinoma, germ cell tumor, and even malignant melanoma. Small cell hematolymphoid neoplasms (like blastic hematolymphoid neoplasms and extramedullary myeloid tumor) may resemble small cell carcinoma, rhabdomyosarcoma, Ewing sarcoma, etc. Plasmacytoma in the head and neck region may resemble neuroendocrine tumors. Thus, a meticulous morphological examination and an adequate immunohistochemistry (IHC) workup may be required. IHC markers may include LCA, CD20, CD3, CD7, Pax5, CD10, Tdt, CD34, AMPO, c-kit (for blastic hematolymphoid neoplasms), CK and EMA (for epithelial carcinoma), S100, HMB45, Melan-A (for melanoma), c-kit, CD30, PLAP, AFP (for germ cell tumors), Desmin, Myogenin, Myo-D1 (for myogenic tumors), Mic-2, EWS-FLI1 (for PNET/ES), others like CD56, synaptophysin, and chromogranin (for neuroendocrine tumors), etc. Be aware that CD99 is expressed in round cell tumors such as PNET/ES and also in blastic lymphomas. Round cell tumors which are negative for LCA, CD3, and CD20 (initial panels employed in most histopathology lab) and express CD99 have been labeled as PNET/Ewing sarcoma. Such cases may be mislabeled as ES/PNET, if additional markers (like Pax5, CD79a, CD10, Tdt, CD34) are not done. Moreover, new entities like early T precursor ALL might be weakly positive or negative for CD3, highlighting the importance of CD7 in such cases for identifying T-cell lineage of these blastic lymphomas (author's experience). One has to be aware of rare spindle cell lesions in nodes like myofibroblastoma, IgG4-related diseases, etc.

The third question may be to differentiate HL from NHL and also further subtyping of HL and/or NHL. Few subtypes of NHL like PTCL-NOS, Lennert's lymphoma, and AITL may resemble classic HL. Granulomas as previously mentioned may be seen in infective conditions like tuberculosis and also in lymphomas such as cHL and PTCL. Occasionally, both HL and NHL may mimic a reactive node. cHL may even mimic NLPHL. More than 95% of HL is of classical subtype, and NLPHL constitutes approximately 5% in our practice, as reported elsewhere [1]. Further subtyping of a hematolymphoid neoplasm is important for the management and prognostication of the patient. The pathological diagnosis of lymphoma is based on morphological interpretation aided by immunophenotypic analysis (either immunohistochemistry or flow cytometry). The ancillary techniques like cytogenetic and molecular analysis provide diagnostic and prognostic information in lymphoma diagnosis and may be important in certain unresolved cases. Many large cell lymphomas have an immune profile of classical HL. These may be labeled as large cell lymphoma with Hodgkin phenotype or else may belong to the category of classical HL—lymphocyte depleted. HL is the common lymphoma subtype seen in practice. It is easily diagnosed based on RS-like cells in a polymorphous background. However, it is one of the most commonly misdiagnosed diseases as differentials vary from a reactive node to other B-cell lymphomas like TCRBCL, NLPHL diffuse variant, and Gray zone lymphoma to T-cell lymphomas like ALCL and PTCL-NOS. Thus, HL is another big waste basket like DLBCL and PTCL-NOS.

Low magnification or scanning objective lens for patterns: Either 1× (expensive lens) or a 2× objective lens is preferred in the diagnosis of HLN. Low magnification gives information about the architecture of the lymph node, different compartments of the node, their size and relationship with each other. The whole mount view of lymph

node is best seen under scanner view with optimum increase in contrast obtained by altering the intensity of light (low) and moving the condenser up and down. Pathologist must spend maximum time on scanner or a low-power view. Most of the times, the diagnosis is established on a scanner view of the whole node. At least it gives a lead and direction, whether one is dealing with reactive lymphoid proliferations or a lymphoma or a metastatic carcinoma. Identifying the pathologic process, that is, focal, multifocal, or predominantly diffuse is very important for the formation of a differential diagnosis of lymphoma. It is good practice to evaluate various componants of lymph node sequentially at scanning magnification namely the capsule, sub-capsular and medullary sinuses, follicles, mantle and marginal zones, interfollicular areas, and medullary cords for abnormalities. There are specific abnormalities that occur in specific compartments, and their recognition permits formulation of a differential diagnosis. Examine all nodes of a given case as one may observe different patterns in different sections. Most lymphomas have a diffuse involvement; however, few may have focal involvement.

Scanner view gives an impression on cellularity, architecture, and color of the node. Architecture is effaced loosely (and commonly). We know nodes have a dynamic structure and hence different nodes as in a case of axillary dissection in carcinoma breast reveal different architecture. Some have a prominent follicular pattern, other might have prominent T zone, and some may have sinus histiocytosis. It does not imply that the architecture is effaced. Absence of secondary follicles does not mean the architecture is effaced. Architecture is effaced as in when nodes reveal something which should not belong there like necrosis, granulomas, metastatic deposits, fibrotic bands, etc. Appearance of nodularity (pseudo-follicular structure or spherical structure), diffuse nature, sclerotic bands, and proliferation centers are best appreciated at scanner and low power. Blue looking node means that the lesion is composed of monomorphic small cells with scanty cytoplasm. Likewise, a pink appearing node means that the lesion is composed of a polymorphous infiltrate of hematopoietic cells and may include neutrophils, eosinophils, histiocytes, immunoblasts, and plasma cells, apart from the atypical lymphoid cells containing more cytoplasm. Blue appearing nodes are classically blastic lymphomas (LL and myeloid sarcomas) and also small cell lymphomas like mantle cell lymphoma, follicular lymphoma, and small lymphocytic lymphoma. Pink looking nodes (polymorphous background and tumor cells with more cytoplasm) include cHL, PTCL, AITL, ALCL, MZL, etc. Nodular looking lymphomas include cHL, NLPHL, FL, MCL, MZL, SLL, etc. while diffuse looking lymphomas include DLBCL, ALCL, cHL, PTCL, MCL, SLL, MZL, plasmacytoma, blastic lymphoma, etc. Tumors with a thick capsule include cHL. Tumors with granulomas include PTCL (Lennert's lymphoma) and cHL. Lymphomas with a rich vasoformative background and high endothelial venules include PTCL and AITL. Tumor cells in PTCL may be small size admixed with large-sized background immunoblasts. Such cases can only be diagnosed with comprehensive T-cell panels including CD2, CD3, CD4, CD5, CD7, CD8 and also PD1, CD23, bcl6, and CD10 (for AITL). T/NK-cell lymphomas need extra markers including CD56, CD57, granzyme, perforin, and TIA-1.

Various patterns in lymphomas: Various patterns like follicular, nodular, mantle, marginal, sinusoidal, Indian file, interfollicular, and starry sky have been described. Different lymphomas have different patterns and similar pattern can be seen in different lymphomas (Table 16.3).

High-power lens for determining the cell size and other cellular features (40× objective lens): Different types of cells occur in compact groups and can be recognized (with higher power of objective lens) on the basis of their color or their locations (e.g., follicles, mantle or marginal zones, interfollicular areas, sinuses, and medulla). The size of tumor cells is usually compared to that of nucleus of endothe-

Table 16.3 Differential diagnosis of various patterns

Pattern seen in lymph node	Differential diagnosis
Nodular/follicular pattern: ball-like structure of one or two layers with follicular center cells	It is seen in follicular lymphoma grade I, II, and IIIA Follicular pattern can be seen due to follicular colonization by mantle cells in MCL or by marginal zone B cells in marginal zone B-cell lymphoma
Pseudofollicular pattern/proliferation centers: pale staining, hypodense, vague nodular structures without color of mantle zone cells, with or without mitotic activity, and tingible body macrophages	Commonly seen in SLL and rarely in lymphoplasmacytoid lymphomas, pseudofollicles being rich in prolymphocytes
Mantle/marginal zone pattern	Mantle cell lymphoma and marginal zone lymphoma, respectively
Nodules with intermingling of layers	Seen in NLPHL; single to multiple large-sized, closely packed spherical structures, also called as progressive transformation of germinal centers (PTGC), with preponderance of small, dark blue lymphocytes (mostly CD20+ small B cells) containing scattered L&H type RS cells (CD20+ large B cells)
Fibrous nodular pattern	Classical HL—nodular sclerosis and, rarely, ALCL may show this pattern
Starry sky pattern: presence of numerous benign histiocytes with abundant clear cytoplasm and phagocytosis	BL and blastic lymphomas (also seen in Kikuchi Fujimoto disease)
Sinus pattern	ALCL, mycosis fungoides, Langerhans cell histiocytosis, marginal zone B-cell lymphoma, etc. Metastasis of carcinoma may show sinus pattern. Subtle sinusoidal pattern in BM trephine is commonly seen in hepatosplenic gamma-delta T-cell lymphoma (CD3+) and SMZL (CD20+) and intravascular large B-cell lymphoma
Interfollicular pattern	PTCL, leukemia, interfollicular-type cHL, etc.
Vascular pattern	Angioimmunoblastic T-cell lymphoma and PTCL-NOS

lial cells or reactive histiocytes. Small-, medium-, and large-sized cells refer to the nuclei, respectively, smaller than, approximately the same size as, or larger than those of the reactive endothelial cell or the reactive histiocyte. Fine cellular details like nuclear configuration (e.g., cleaved cells), features of nucleoli, and cytoplasmic characteristics are visualized best at high magnification. Small-cleaved cell lymphomas include follicular lymphoma (FL) and mantle cell lymphoma (MCL) while large cell lymphomas commonly are diffuse large B-cell lymphoma (DLBCL), anaplastic large cell lymphoma (ALCL), plasmablastic lymphoma (PBL), etc. BL are classically composed of monomorphic intermediate-sized cells with numerous tingible body macrophages and mitoses in the background.

The cellular microenvironment also contributes to differential diagnosis of lymphoma. The background of plasma cells, eosinophils, neutrophils, and histiocytes is mostly seen in cHL, PTCL-NOS, AITL, etc. Increased histiocytic cells in the background are seen in classical HL, T-cell histiocyte-rich B-cell lymphoma, PTCL, or AITL. For mantle cell lymphoma, scattered pink histiocytes between sheets of monomorphic small-cleaved lymphocytes may be a helpful diagnostic clue (with the absence of nucleolated cells). Clusters of epithelioid histiocytes may be seen in T-cell lymphomas (Lennert's lymphoma) and cHL. Tingible body macrophages containing cellular debris give a starry sky pattern in BL and in lymphoblastic lymphoma (not to confuse with normal T zone mottling seen in reactive nodes or in dermatopathic lymphadenitis).

Benign Versus Malignant Lymphoid Proliferations

Diagnosis of lymphoma is mostly based on the examination of a H&E stain slide. IHC stains can further substantiate morphological findings (and help it further subtype). Many benign diseases of lymph node like bacterial and viral infections including HIV, toxoplasmosis, Kikuchi Fujimoto disease, dermatopathic lymphadenitis, sinus histiocytosis, lymphadenopathy related to collagen vascular diseases, and drugs and also immune reactions (like autoimmune lymphoproliferative syndrome) might mimic a hematolymphoid neoplasm. These nodes may be small to large in size and have a partial effacement of architecture with a prominent follicular hyperplasia and/or paracortical T-zone expansion. Immunoblasts may be prominent in the follicular and paracortical zones and may be confused with RS cells and may express CD45, CD20, and CD30. Kimura disease, Castleman disease, and dermatopathic lymphadenitis may resemble HL while Kikuchi Fujimoto disease may resemble DLBCL/BL. Another common diagnostic dilemma is differentiating reactive follicular hyperplasia from follicular lymphoma. Reactive follicular hyperplasia has classical morphological features. It shows preservation of nodal architecture and low density of follicles, which are variable in size and shape with interfollicular zone. The follicles have sharp demarcation of germinal center from that of mantle zone. Germinal centers will show polarization, brisk mitosis, and tingible body macrophages. Many a times reactive follicular hyperplasia may mimic with follicular lymphoma and IHC with BCL2 may or may not help solve this issue. BCL2 staining within the germinal centers favors a diagnosis of follicular lymphoma, though the FL grade 3 may be negative for bcl2 expression (as seen in cutaneous FL). In reactive germinal center, bcl2 is negative in centrocytes and centroblasts (B cells); however, CD4+ T-helper cells within germinal centers may express bcl2. In situations where CD4+ T cells (bcl2+) predominate within the germinal center, it may mislead to a diagnosis of follicular lymphoma. These CD4+ T-helper cells within germinal centers may give rise to AITL (in addition to T-cell markers, they express CD4, CD10, bcl6, PDI) and CD21/CD23 highlights an extra germinal center follicular dendritic cell proliferations.

Common infectious agents associated with various lymphomas include Epstein–Barr virus (Burkitt lymphoma, cHL, extranodal T/NK-cell lymphoma, angioimmunoblastic T-cell lymphoma, primary CNS large B-cell lymphoma, EBV-positive DLBCL, post organ transplant lymphomas), HTLV-1 (adult T-cell leukemia lymphoma), HIV (DLBCL, BL, plasmablastic lymphoma), hepatitis C virus (lymphoplasmacytic lymphoma), human herpes virus 8 (primary effusion lymphoma, multicentric Castleman disease).

IgG4-related disease is a newly recognized fibro-inflammatory condition characterized by several features: a tendency to form tumefactive lesions in multiple sites; a characteristic histopathological appearance; and often-elevated serum IgG4 concentrations [8]. Such patients may present to any of the following specialties including pathology, rheumatology, gastroenterology, allergy, immunology, nephrology, pulmonary medicine, oncology, ophthalmology, and surgery. Diagnosis of IgG4-related disease is based on the combined presence of the characteristic histology (triad of dense lymphoplasmacytic infiltrate, a storiform pattern of fibrosis, and obliterative phlebitis) and increased numbers of $IgG4^+$ plasma cells. In tissues, dense diffuse infiltrates of $IgG4^+$ plasma cells that number >50/hpf are reportedly highly specific. $IgG4^+/IgG^+$ plasma cell ratio of >40% as a comprehensive cutoff value in any organ is also very specific of IgG4RD.

16.4.6.2 Immunophenotyping and Lymphoma

The different methods of immunophenotyping that yield similar information are IHC, FCM, and immunofluorescence, with the former two being mandatory for any laboratory reporting HLN. It may be expensive to do both IHC and FCM in each case of lymphoma. FCM is best done for blood/bone marrow/fluids, while IHC is optimal for lymph nodal and extranodal lesions. Both the techniques are complementary. FCI may also be

done on FNAC of lymph nodes or tissue homogenate from lymph node. Few centers use both FCI and IHC in the diagnosis of lymphomas. FCI is performed as a panel rather than an individual marker. Furthermore, understanding of the normal staining pattern and cross-reactions of an antibody is crucial for the correct interpretation and diagnosis. There are markers that work better on IHC like cyclinD1 and other markers that are more popular on FCI like CD200, etc. All labs doing IHC and FCI must have a stringent internal quality control program and also participate in a proficiency-testing (external quality assurance) program. Each new lot of antibody needs to be verified, as required, before it is put in to the diagnostics. All laboratories should preferably be accredited and conform to international quality standards (ISO:15189:2012).

Immunohistochemistry

Of all the available ancillary techniques, IHC is a more widely available tool for diagnosing HLN. FCI of lymphomas as B-, T-, or T/NK-cell type is not straightforward as many of them have a polymorphous population in the background. There are more than 250 CD markers available today and approximately 40–45 being routinely used in IHC labs for the diagnosis of HLN (Figs. 16.5, 16.6, 16.7, 16.8 and Tables 16.4, 16.5, 16.6, 16.7, 16.8, 16.9, 16.10, 16.11, 16.12). It is important to understand the reactions of these markers with respect to different normal

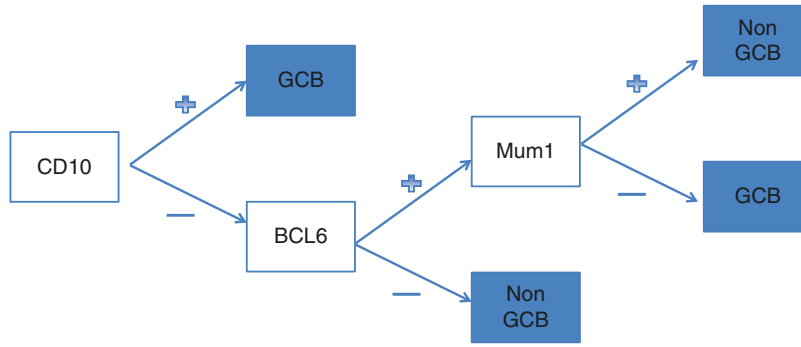

Fig. 16.5 Hans algorithm

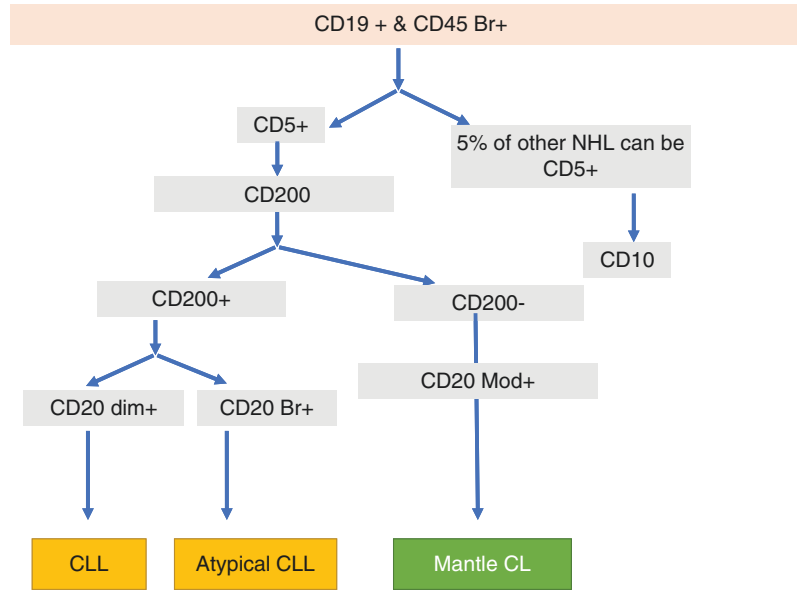

Fig. 16.6 Differentials in mature B-cell neoplasms based on bright CD45 and CD19 positivity

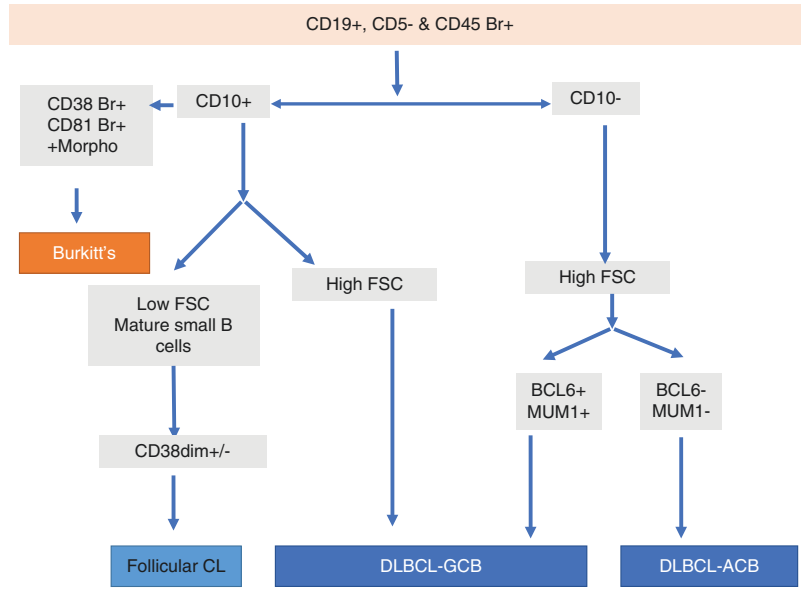

Fig. 16.7 Mature B-cell neoplasms based on bright CD45 and CD19 positivity

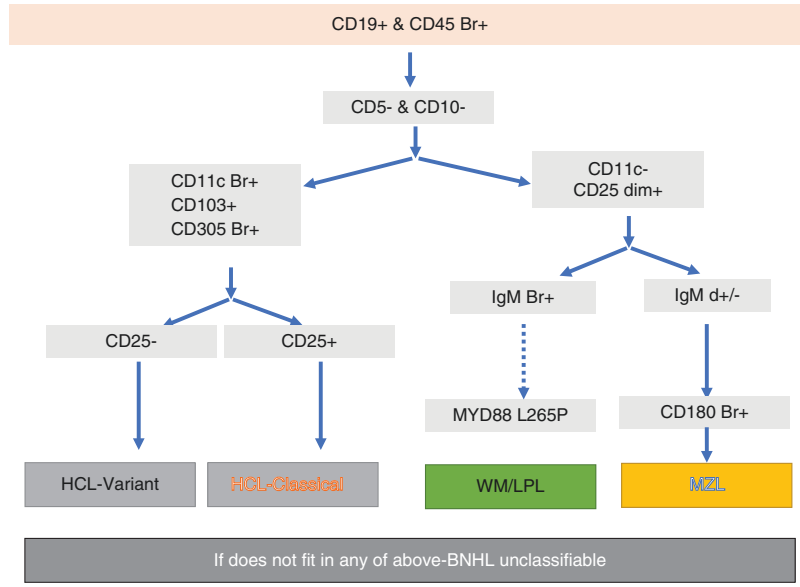

Fig. 16.8 Mature B-cell neoplasms based on bright CD45 and CD19 positivity

Table 16.4 Essential markers for a hematopathology laboratory

LCA, CD20, CD3, CD5, CD23, cyclinD1, Mib1, CD10, bcl6, bcl2, CD43, CD15, CD30, Alk1, Pax5, Tdt, CD34, CD138, MPO, Mum1, EBVLMP1/EBER (ISH)

cells in the lymphoid and non-lymphoid organs. An example is of bcl2 reaction in a normal lymph node. Bcl2 is commonly used in the differential diagnosis of follicular lymphoma from follicular hyperplasia. In a normal node, bcl2 is expressed by mantle cells, marginal zone cells, interfollicular T cells, and in T cells (CD4+) present within the germinal centers. Similarly, Pax5 is a B-cell marker with the strongest expression in mantle zone cells, weak to moderate in germinal center C cells, weak in RS cells (in CHL), and negative in marginal zone B cells, plasma cells, and T cells. CD138 is a marker for plasma cells and is also expressed in epithelial

Table 16.5 Comprehensive panel of antibodies as a lymphoma panel

B-cell lymphoma panels: CD20, CD79a, CD19, CD23, CD10, MYC, CD5, BCL2, BCL6, Ki67, IRF4/Mum1, CD21, CD23, CD35, cyclin D1, Pax5, Tdt, CD138, kappa and lambda light chains, CD43, p21, Annexin A1, CD123
Hodgkin lymphoma panel: LCA, CD3, CD20, CD15, CD30, Pax5, ALK1, IRF4/Mum1, EBV-LMP1, EBE-EBER (ISH), Oct2, Bob1, EMA, CD57
T/NK-cell lymphoma panel: CD2, CD3, CD4, CD5, CD7, CD8, CD56, Ki67, CD23, CD10, CD25, CD30, Alk1, Tdt, CD56, CD57, Perforin, Granzyme, TIA-1, PD1, CD123
Miscellaneous: C-kit, MPO, CD33, HLADR, CD1a, CD163, CD61/CD41, CD235, CD71, etc.
Other markers: CK, EMA, S-100, HMB45, Melan A, Desmin, Mic2, FLI1, MyoD1, Myogenin, Synaptophysin, Chromogranin, ER, PR, Cerb b2

Table 16.6 Different lymphoma subtypes and panels

DLBCL: CD10, bcl6, Mum1, Mib1, bcl2, MYC
Plasma cell neoplasms: CD79a, CD38, CD138, kappa/lambda light chains, CD56, EMA, Mum1, cyclinD1, CD19, C-kit
Small cell lymphomas: CD5, CD23, cyclinD1, bcl6, CD10, CD43, bcl2
Burkitt lymphoma: bcl2, MiB1, CD10
Lymphoblastic lymphoma: CD3, CD99, TdT, Pax5, CD79a, CD10, CD4, CD8 (for B-LL, CD79a and CD19 are better markers as the blasts might be negative for LCA and CD20)
Granulocytic sarcoma: CD43, MPO, cKit, CD34
ALCL: CD30, ALK-1, EMA, CD3, CD4, CD8, CD20
PTCL/AITL: CD3, CD2, CD5, CD7, CD56, CD30, ALK1, CD23, CD10, CD4, CD8, PD1, Tdt
Suspected NK cell lymphoma: CD3, CD56, CD57, EBER (others: CD8, TIA-1, granzyme B)
Histiocytic differentiation: PGM1 (CD68), CD163, CD1a
Dendritic cell: CD21, CD23, CD35, S-100
cHL: LCA, CD3, CD20, Pax5, CD15, CD30, Alk1, EBER ISH, EBV LMP1
NLPHL: CD20, CD3, CD56, Oct2, Bob1, CD23. CD30, EBER, EBVLPM1
Cutaneous lymphomas: CD3, CD20, CD4, CD8, CD5, CD7, CD10, CD30, BCL2
EBV-related lymphomas: EBER by in situ hybridization

cells. CD10 is a marker used in hematolymphoid neoplasms where it is expressed in precursor B-cell ALL, DLBCL, FL, BL, AITL, and also in benign lymphoid cells like hematogones and also in neutrophils and stromal cells. CD10 expression in neutrophils is used as an internal control in FCI.

Large list of CD markers is available, and newer ones are added on a regular basis. Histopathologist shall decide the panel based on morphology and his/her expertise. Most of the time, a provisional diagnosis for the lesion is established on morphological examination. Accordingly, a panel of antibodies is applied to confirm this and further subtype the neoplasm. The panel also helps in identifying predictive markers (CD20+ B-cell lymphoma may receive rituximab therapy) and prognostic markers (CD38 and Zap 70 in CLL). IHC may also help in tricky situations like in differentiating a benign from a malignant proliferation (follicular hyperplasia versus follicular lymphoma).

It is important to select and understand the cells of interest and interpret the IHC patterns accordingly. There are neoplasms where tumor cells are very scanty (may be <1% tumor cells), as seen in classical HL. Here the tumor cells are RS cells while the majority of the cells in the background (lymphocytes, plasma cells, histiocytes, neutrophils, and eosinophils) are believed to be a response to the tumor. Thus, IHC is evaluated accordingly. HL has overlapping morphological features with T-cell histiocyte-rich B-cell lymphoma and peripheral T-cell lymphoma (NOS). Selection and interpretation of cells of interest is crucial and may be based on morphological and/or immunophenotypic evaluation.

Table 16.7 Differential diagnosis of HL and NHL resembling Hodgkin lymphoma

Lymphoma	Principle site of involvement	Histology	Tumor cell immunophenotype
cHL	Peripheral lymphadenopathy, mediastinal mass, spleen, Para-aortic LN	RS cell is a constant feature. Sinus infiltration usually absent. Sheets of RS cells can surround area of necrosis in syncytial variant of HL	CD45−, CD30+, CD15+/−, PAX5+, ALK−. EMA−, EBV+/−,BOB1,OCT2−/+,CD20+/− Background is T cell (CD3) rich
NLPHL	Peripheral lymphadenopathy, mediastinal mass, spleen, and bone marrow involvement are rare	Usually a nodular or a diffuse infiltrate with small lymphocytes, histiocytes with scattered tumor cells (RS like, popcorn, L&H cells etc)	CD45+, CD30, CD15−, CD20+, BCL6+, OCT2+, BOB1+, EMA+/−, ALK1−. EBER negative. PTGC nodules rich in CD20+ small B cells admixed with CD20+ L&H cells T-cell rosetting around L&H cells: CD3+, CD4+, CD57+, PD1+
ALCL	Peripheral lymph node, extranodal sites (skin, gastrointestinal tract, bone)	Hallmark cells having eccentric, horseshoe, kidney, or embryo shaped nuclei with eosinophilic region near nuclei seen in sheets. Sinus infiltration is common. RS cells are rare	CD45+/−, CD30+, ALK+/−, CD15-,CD3+/−,CD4+,EMA+/−
PTCL-U	Generalized lymphadenopathy, spleen, skin	Some cases have numerous large cells, which mimic RS cells. Surrounding cells show spectrum of medium and small atypical cells	CD45+, T-cell markers+ however may show loss of any of T-cell antigens (CD7, CD5, CD3, or CD2). Ratio of CD4/CD8 is altered. CD30 may be focally positive while Tdt is negative B-cell markers negative (rarely CD20+ PTCLs have been described)
T-cell/histiocytic-rich B-cell lymphoma (T/HRBCL)	Peripheral lymphadenopathy, but bone marrow, liver, and spleen are frequently involved at the time of diagnosis	A small number of large cells (around 10% neoplastic B cells) seen surrounded by bland-looking histiocytes and small lymphocytes (T type). Tumor cells do not form aggregates or sheets	CD45+, CD20+, BCL6+, BCL2+/−, EBV+, EMA+/− and CD30−, CD15−, CD138− and T−. Background T cells CD3+ along with histiocytes. The T cells are CD3+,CD57−,and PD1−
Primary mediastinal large B cell lymphoma	Mediastinal mass (thymus), spread to kidneys, adrenals, liver; common in young females	Tumor cells are medium-sized to large cells with abundant pale cytoplasm. Pleomorphic and/or multilobated nuclei may resemble RS cells. Bands of collagen surround the individual and small groups of cells (alveolar fibrosis)	CD45+, CD20+, CD23+/−, CD30+ (+ in about 80% cases), MUM1+,BCL6+/−,BCL2+/−,CD10−

Lymphoma Diagnostic Panels for IHC

Subtle diagnoses that are easily missed on the basis of standard H&E-stained sections include interfollicular HL, sinusoidal infiltration by ALCL, partial nodal involvement by follicular lymphoma or in situ lymphomas, etc. MCL has many morphological variants like in situ type, classical lymphocytic type, blastoid type, and rare polymorphous type. Thus, MCL may have a morphological differential with FL Grade 1 (or SLL) and also a blastic lymphoma. These should be recognized by careful analysis at light micros-

Table 16.8 IHC and genetic features of common mature B-cell NHL

Mature B-cell NHL	CD5	CD23	CD10	CD43	BCL2	Annexin A1	CyclinD1	Genetic abnormality
CLL/SLL	+	+	–	+	+	–	–/+	13q deletion (commonest), trisomy12, deletion of 11q22–23(ATM), and 17p13(p53)
MCL	+	–/+	–/+ in blastoid variant	+	+	–	+	t(11;14)(q13;q32) between IGH@ and/ cyclinD1(CCND1) gene
LPL (IgM+)	–	–	–	+	–	+	–	No specific abnormalities, del6q on BM and rarely t(9;14)(IGH/PAX5)
FL	–	–	+ with BCL6+	–	+	–	–	t(14;18)(q32;q21) between IGH@/ BCL2
DLBCL (germinal center/ GC type) and (activated B cell/ ABC type)	–/+	–	+(with BCL6+ in GC)/– (with BCL6– in ABC)	–/+	+	–	–	t(14;18)/IGH@/ BCL2, abnormality of 3q27(BCL6 gene), gain of 9p and 12q12
Burkitt lymphoma (Mib-1-100%)	–	–	+	–	–	–	–	t(8;14), t(2;8), and t(8;22):Detection by MYC break-apart by FISH
HCL (CD11c, CD25, CD123, CD103)	–	–	–	+	+	+	+	No specific abnormality
SMZL (IgM+, IgD+)	–	–/+	–	–	+	–	–	Allelic loss of 7q31–32
Mucosa-associated lymphoid tissue lymphoma	–	–/+	–	–/+	+	–	–	t(11;18)(q21;q21) (API2/MALT1) t(1;14), t(14;18), and t(3;14) deregulation of BCL10, MALT1, and FOXP1, respectively

copy, but a small panel of antibodies will be required to correctly subtype these lymphomas. Though there is no defined list for minimal markers, a laboratory must have adequate B, T, myeloid, and other common markers for subtyping of HLN. Attached are the tables with suggested list of the common antibodies used in lymphoma diagnosis (Figs. 16.5, 16.6, 16.7, 16.8 and Tables 16.4, 16.5, 16.6, 16.7, 16.8, 16.9, 16.10, 16.11, 16.12). It is important to mention here that many carcinomas or round cell tumors might mimic a HLN and hence smaller panels can be misleading.

Specialized centers should aim to perform a comprehensive diagnostic workup as per WHO 2017 Classification of HLN. Smaller labs might have lesser markers in their armamentarium (Table 16.4) and, however, can refer cases to specialized centers for additional IHC markers, as and when need arises. Though there is no list of

Table 16.9 IHC and genetic features of common mature T-cell lymphoma (TCL)

T-cell neoplasm	CD3	CD5	CD4	CD8	CD30	TCR	NK (CD16, CD56)	Viral infection	Genetic abnormalities
T-PLL	+	–	+/–	–/+	–	αβ	–/+	–	inv(14), trisomy 8q
T-LGL	+	–	–	+	–	αβ	+/–	–	No specific abnormality
NK LGL	–	–	–	+	–	–	+	EBV+	No specific abnormality
ATLL	+	+	+	–/+	–/+	αβ	–	HTLV1	No specific abnormality
Extranodal NK/TCL, nasal type	–/+	–	–	–	–	–	+	EBV++	Deletion (6) (q21;q25) commonest abnormality
Hepatosplenic γδ TCL	+	–	–	–/+	–	γδ	+/–	–	iso(7q) abnormality
Enteropathic type TCL	+	+	–	–/+	+/–	–	–	–	No specific abnormality
Subcutaneous panniculitis like TCL	+	+	–	+	–	αβ	–	–	TCR gene rearrangement
Primary cutaneous γδ TCL	+,+/–					γδ	+	–	No specific abnormality
Mycosis fungoides	+	+	+	–	–	αβ	–	–	No specific abnormality
PTCL-NOS	+/–	+/–	+/–	–/+	–/+	αβ > γδ	–/+	EBV–/+	Complex karyotype
Angioimmunoblastic	+	+	+/–	–	–	αβ	–	EBV+/–	No specific abnormality
Primary systemic ALCL (ALK+/–)	+/–	–/+	+/–	–/+	+	αβ	–	–	t(2;5) NPM/ALK
Cutaneous ALCL (ALK–)	+/–	–	+	–	+	αβ	–	–	No translocation involving ALK gene

minimal markers for the diagnosis of lymphomas, two levels (based on number of antibodies used) of antibodies may be suggested (Tables 16.4 and 16.5): first level of best laboratory practice where all available markers are available and second level where bare essential markers are available to make a diagnosis in the vast majority of cases.

Various lymphoma subtypes may look similar on morphology and may also mimic non-neoplastic lesions or other tumors like a carcinoma, melanoma, germ cell tumor, or even a round cell tumor. Moreover, a pathologist will not know what is going to be on his/her reporting table next. Few common morphological mimics include HL and ALCL or a T-cell histiocyte-rich B-cell lymphoma, nasopharyngeal carcinoma, and DLBCL, plasmablastic lymphoma and malignant melanoma, and lymphoblastic lymphoma and any other round cell tumor. Many of B-cell lymphomas like plasmablastic lymphomas might not express CD20, thereby highlighting the requirements of more extensive panels like CD138, Mum1, and CD79A. Moreover, ALK1, which is classically expressed in ALCL, may also be expressed in large B-cell lymphomas, and cyclinD1 may be expressed in hairy cell leukemia and plasma cell neoplasm, apart from mantle cell lymphoma. Few MCL might not express cyclinD1 where we need additional stains including Sox11.

Small-cleaved cell lymphomas (monomorphic cells) include differential diagnoses of MCL and FL (grade 1). MCL generally has a

Table 16.10 Differentials considered for mature B-cell neoplasms based on the following groups

CD5/CD10	Differential diagnosis
CD5+ CD10−	1. CLL: CD23, CD19+, FMC7−, CD22, CD20, and CD79b are weak positive 2. MCL: CD23− or only weak positive, CD19+, FMC7+, CD22, CD20, and CD79b are positive but confirmation is by either cyclinD1 positivity by IHC or t(11;14)(q13;q32) by cytogenetic studies 3. B-PLL: In comparison with CLL, prolymphocytic transformation shows decreased staining for CD5 and increased staining for CD20 and acquisition of FMC7 4. Marginal zone lymphoma (MZL): 5% MZL may be CD5+ and difficult to differentiate from CLL 5. Lymphoplasmacytic lymphoma (LPL): Approximately 5% of LPL are CD5+ and need to be distinguished from MZL and other B-cell lymphoma with plasmacytoid differentiation 6. DLBCL: A subset of DLBCL is CD5+ and it is difficult to differentiate between de novo DLBCL and Richter transformation in CLL
CD5− CD10+	FL and DLBCL represent the most frequent CD10+ and CD5− mature B-cell NHL, followed by Burkitt lymphoma. The lymph node biopsy and cytogenetic study for t(14;18) and c-myc gene rearrangement (BL) may solve the dilemma. It is known to be present in B-ALL Uncommonly, CD10 can be seen in hairy cell leukemia, MCL (CD5+ and blastic variant), and MZL. Very rarely CD10 is also present in T lymphoma like precursor T-cell lymphoblastic lymphoma and angioimmunoblastic lymphoma
CD5− CD10−	The common group involved under this group is DLBCL (may be activated cell type), SMZL, HCL, and LPL. Very rarely CD5− MCL and CD10− FL should be considered Additional marker like CD25, CD103, CD11c, and CD123 by flow cytometry helps in differential diagnosis of SMZL, HCL, and HCL variant HCL: CD25, CD103, CD11c, and CD123+ with annexin a1, DBA.44. and cyclinD1+ (maybe) on trephine biopsy HCL-variant: CD25–CD123− with CD103+ CD11c+ with annexin a1− and DBA.44+ on trephine biopsy SMZL: CD180 bright, CD25dim, CD103– and CD11c+/–, cyclinD1–, and DBA.44+/− on trephine biopsy
CD5+ CD10+	Very rarely this pattern is seen in MCL, DLBCL, and FL

Table 16.11 Differentials considered for mature T-cell neoplasms based on the following groups

CD4/CD8	Differential diagnosis
CD4+ CD8−	Sézary syndrome/cutaneous T-cell lymphoma, T-cell PLL, adult T-cell leukemia/lymphoma (ATLL), ALCL (rarely present as leukemic phase), angioimmunoblastic T-cell lymphoma, PTCL-U, and rarely CD4+ T large granular leukemia (LGL)
CD4− CD8+	T-cell LGL is the most frequent CD8+ T-cell neoplasm. Other T-cell lymphomas with CD8 positivity are hepatosplenic T-cell lymphoma, NK cell LGL, CD8+ PTCL-U. and rarely T-cell PLL
CD4+ CD8+	Dual expression of CD4 and CD8 is usual in T-ALL (TdT or CD34+) and unusual in mature T-cell lymphoma. Though, double positivity for CD4 and CD8 can be seen in T-PLL with surface CD3 positivity and lack of TdT and CD34. Very rarely, ATLL and LGL may show this co-positivity
CD4− CD8−	Enteropathic T-cell lymphoma, hepatosplenic gamma-delta T-cell lymphoma, PTCL-U, and rarely NK/T-cell lymphoma nasal type with T-cell type lymphoma

classical monomorphic pattern of involvement by small-cleaved cells admixed with pink histiocytes and hyalinized capillaries, and absence of nucleolated cells. MCL and MZL can have various patterns including nodular, diffuse, and also mantle/marginal zone growth pattern. Mantle cell lymphoma blastic variant may resemble lymphoblastic lymphoma. Here additional stains like Tdt, CD34, CD5, cyclinD1, and Sox11 are helpful. NLPHL classically presents as single or multiple large-sized nodules (progressive transformation of germinal centers) with the presence of L&H cells expressing LCA and CD20 strongly.

Table 16.12 Useful markers (reagents) for acute leukemia panel

Backbone marker	CD45 (leucocyte common antigen)
Markers of immaturity	CD34, dim CD45, TdT
Myeloid lineage	Cytoplasmic MPO, CD117, CD13, CD33, CD15, CD11b
Monocytic lineage	CD4, CD14, CD11b, CD11c, CD36, CD64, CD65, CD66, HLADR
Megakaryocytic	CD41, CD61, CD36
B lymphoid	CD10, CD19, CD22, cytoCD79a, CD20
T lymphoid	Cytoplasmic CD3, surface CD3, CD1a, CD2, CD4, CD5, CD7, CD8
Plasmacytoid dendritic	CD123, CD302, 303 (CCD4, CD56, CD43)
Erythroid	CD235a, CD71, CD36
Leukemia-associated immunophenotypes (LAIPs)	
AML	CD7, CD19, CD56
B/T-ALL	CD13, CD15, CD33, CD117
Markers for B-cell chronic lymphoproliferative disorder (lymphoma) panel	
General	CD19, CD20, CD22, cyto79a, CD79b, CD23, FMC7, kappa, lambda
Markers useful for further subclassification in B NHL	
CLL/MCL	CD5, CD23, CD43, CD200, CD38, Zap70, light chain restriction
FCL/BL/DLBCL	CD10
HCL	CD11c, CD25, CD103, CD123
Markers for T-cell chronic lymphoproliferative disorder (lymphoma) panel	
General	CD2, CD3, CD4, CD5, CD7, CD8, TCRαβ, TCRγδ
Markers useful for further subclassification in T NHL	
ATLL/CTCL	CD25, CD26
AITL	CD10
ALCL	CD30
LGL	CD56, CD57
EATCL	CD103
NK cells	CD16, CD56, CD57, CD94, CD158
Markers for plasma cell dyscrasia panel	
General	CD138, CD38, CD19, and cytoplasmic kappa and lambda
Abnormality associated	CD56, CD20, CD28, CD117; also loss of CD27 and CD81

Small non-cleaved cell lymphomas (monomorphic small- to intermediate-sized cells) with high mitoses and apoptosis is a classical morphology of BL. BL has a common differential with DLBCL, and there is an entity with overlapping features labeled as high-grade NHL, with features intermediate between BL and DLBCL (an entity deleted in new WHO 2017 Classification of HLN). Similarly, mediastinum, which is a common site of HL (nodular sclerosis pattern), primary mediastinal large B-cell lymphomas (PMLBCL) and DLBCLs, also has a neoplasm with overlapping features between HL and DLBCL labeled as high-grade B-cell lymphoma with features intermediate between HL and DLBCL (a type of gray zone lymphoma). PMBCL is commonly seen in middle-aged to elderly women and has a classical morphology and immunophenotypic pattern.

PTCL is a common subtype of T-cell lymphomas and should be differentiated from ALCLs, AITL, and T-lymphoblastic lymphomas. T-LL must be differentiated from extramedullary myeloid tumors, biphenotypic acute leukemias and also from early T-precursor ALL. ALCL need to be differentiated from Alk-negative ALCL, classical HL, mastocytosis, deposits of carcinoma, and a germ cell tumor (all may express CD30).

DLBCL is the commonest subtype of lymphoma and is considered a wastebasket. They are

aggressive lymphomas with considerable clinical, biologic, and pathologic diversity and reflect the functional diversity of the B-cell system and multiple pathways of transformation. Using a multi-modality approach, the updated WHO 2017 Classification has defined some new subgroups, including DLBCLs associated with age groups or anatomic sites. Thus, DLBCLs have broadly been divided into various subtypes like DLBCL, NOS (primary DLBCL of the CNS, primary cutaneous DLBCL, leg type, intravascular large B-cell lymphoma), TCHRBCL, EBV-positive DLBCL, DLBCL associated with chronic inflammation, PMLBCL, ALK-positive large B-cell lymphoma, PBL, PEL, MCD, and B-cell unclassifiable between cHL and DLBCL. Entity B-cell unclassifiable between BL and DLBCL (gray zone lymphomas) has been deleted in new WHO 2016 Classification of HLN. Although DLBCL is curable with standard chemoimmunotherapy (R-CHOP), over 30% of patients with advanced stage disease experience refractory disease or progression. To differentiate the good DLBCL from the bad DLBCLs, gene expression studies were conducted, and they indicated that DLBCL is a heterogeneous disease entity, as cell-of-origin studies reveal at least three distinct subtypes: primary mediastinal, activated B-cell (ABC), and germinal center B-cell (GCB) types [9]. Genomic profiling has been translated to IHC and by using different algorithms, most commonly Hans algorithm (Fig. 16.5), we can differentiate ABC from GCB subtype [10]. Both subtypes express CD10. GCB express BCL6, while any tumor expressing MUM1 is of ABC subtype. Thus, MUM1 is a bad prognostic marker in DLBCLs.

Laboratory Approach in Case of Large Cell Lymphoma

Most labs have a policy to use a smaller primary panel based on morphology and do a more elaborate secondary panel depending on the findings of primary panel. Others might prefer doing a comprehensive panel upfront, saving turnaround time. Most of these large cell lymphomas are of B-cell type; however, a few of these may be ALCL (T/Null-cell type) or other subtypes. To begin with, three most important markers are (a small primary panel) LCA, CD3, and CD20 in a case suspected to be a large cell lymphoma. In case the tumor cells express CD20, the lab may do secondary panel (depending upon morphology), which may include at least Mib1, CD10, and bcl2 to differentiate DLBCL from BL, and cyclinD1 may be used to differentiate mantle cell lymphoma (pleomorphic variant). Likewise, additional markers like CD10, bcl6, and Mum1 may be required to differentiate good subtype GCB from bad subtype ABC of DLBCL [10]. Newer entities like double-expressor lymphoma (DEL) and double-hit lymphoma (DHL) have been identified in the recent WHO 2017 Classification of HLN [2]. MYC (traditionally a FISH marker) protein has been introduced as an IHC stain. Approximately 30–50% of cases of DLBCL express MYC protein (by IHC) in >40% tumor cells and show BCL2 mutations in >50% cells (in 20–35% such cases) and are labeled as DEL. These are generally ABC subtype and have a bad prognosis. These cases of DEL may be further taken up for FISH studies for MYC/BCL2/BCL6 mutations. Cases of DLBCL expressing MYC by FISH are approximately 5–15%, and these are generally associated with BCL2/BCL6 mutations and are labeled as DHL or Triple Hit Lymphomas (THL) [2]. Thus, in a new case of DLBCL, one may do IHC (MYC, BCL2, BCL6) and follow Hans algorithm. FISH (MYC, BLC2, and BCL6) is not mandatory in all cases of DLBCL and, however, may be attempted in situations like DLBCL with GCB subtype, DLBCL with high-grade morphology and DLBCL with >40% MYC on IHC. Entity of BCLU (intermediate between BL and DLBCL) has been eliminated in new WHO 2017 Classification. High-grade B-cell lymphoma, NOS (HGBL, NOS), is a new entity in WHO 2017 Classification, and it encompasses high-grade B-cell lymphoma with BCLU-like morphology and IHC or high-grade B-cell lymphoma with blastoid morphology but lacks MYC and BCL2, and/or BCL6 rearrangements. Thus, in order of severity, HGBL with MYC, BCL2, and BCL6 rearrangement are the worst subtype followed by DHL/THL (by FISH) and DEL (by IHC). To sum up how to

workup a case of DLBCL, one may do only two stains CD3 and CD20, or else an extensive IHC panel including CD3, CD20, MIB1, BCL6, MUM1, CD10, MYC, BCL2, CD30, CD5, cyclinD1, Alk1, CD138, EBER and also FISH for MYC, BCL2, and BCL6.

Similarly, WHO 2017 Classification classifies mantle cell lymphoma into three subtypes. Classical MCL is unmutated subtype, expresses SOX11, is a lymph nodal disease, may involve extranodal sites too, may be blastoid and pleomorphic in morphology, and may have mantle cell zone growth pattern. Another subtype is leukemic non-nodal MCL which is a mutated subtype, SOX11 negative, involves peripheral blood, bone marrow, and spleen, is indolent, is rarely associated with TP53 mutations, and can behave aggressively. The third rare subtype is in situ MC neoplasia where we find cyclinD1-positive cells in inner MC zone [2].

Similarly, dendritic, interdigitating, and histiocytic cells and immunodeficiency-related neoplasms are not uncommon and need an immunomorphological approach. Classical HL needs Pax5 (weak cytoplasmic positivity), EBER (or EBVLMP1) (almost all cases express EBV) in addition to LCA, CD30, CD15, CD3, and CD20. Other situations where NLPHL is close differential, additional markers CD23, CD4, CD8, CD56, Oct2, Bob1, GATA3, etc. may be employed.

Basic IHC panel for lymphoma diagnosis includes LCA, CD20, and CD3. For subtyping, additional markers are required as shown in the tables. Additional panel for other circumstances (based on morphological impression):

Variation in IHC Expression May Help in Interpretation of Hematolymphoid Neoplasms

Aberrant expression by B cells: Co-expression of CD20 (B-cell marker) with CD5 (T-cell marker) may suggest a neoplastic proliferation, though a tiny population of CD5 expressing B-cells has been described in the peripheral blood of normal healthy adults. Expression of cyclinD1 is always abnormal and suggests mantle cell lymphoma, though it can be expressed in hairy cell leukemia and plasma cell neoplasm also.

Aberrant expression by T cells: One can identify abnormal T/NK cells on IHC or FCI if there is

1. Lack of expression of pan T-cell markers like CD7, CD5, CD3, or CD2 (normal NK cells are CD5−).
2. Abnormal intensity of expression of antigen as compared to normal T/NK cells.
3. Aberrant expression of non-T/NK-cell-associated antigen.
4. Abnormal CD4/CD8 ratio or CD4 and CD8 double-positive or negative T cells.
5. Predominance of gamma-delta T cells (double-negative T cells). Normally these are less than 2% of all T cells.
6. The presence of CD1a and Tdt indicates the precursor lymphoblastic lymphoma. CD10 expression can be seen aberrantly in T-cell lymphoma either in T-LL or angioimmunoblastic T-cell lymphoma.

It is important to note that LCA may be negative in few of the HLN like T-LL, B-LL, plasmablastic lymphoma, classical HL, plasma cell neoplasms, etc. BCL2 has a limited role in lymphoma diagnosis. Its expression is seen in normal T cells, marginal zone B cells, mantle zone B cells. Its main use is in differentiating Burkitt lymphoma (BCL2−) from DLBCL (BCL2+) and also in differentiating follicular lymphoma (Bcl2+) from follicular hyperplasia (BCL2−) (best done on morphology).

IHC has an advantage that the histologically abnormal cells can be seen on microscopy to express or lack a particular marker. It may supplement the information generated by FCM and may be the only investigation when FCI is not available. Representative paraffin blocks may be selected for IHC staining. When selecting panels for IHC, it is important to include antibodies that are expected to give negative as well as positive results.

Indian data shows [11, 12] distribution of various NHL subtypes in India. Follicular lymphoma and mantle-cell lymphoma are less common in India compared to Europe and the USA. Peripheral T-cell lymphomas and T/NK-cell lymphomas of

nasal type, which are common in many other Asian countries, are also less prevalent. T-cell lymphoblastic lymphoma and anaplastic large T/null-cell lymphoma are more prevalent in India. In this study, B-cell lymphomas formed 79.1% of the NHLs, whereas T-cell lymphomas formed 16.2% of the NHLs. DLBCL was the most common subtype (34% of all NHLs). FL, B-cell SLL, MCL, and MZL (including MALT lymphomas) amounted to 12.6%, 5.7%, 3.4%, and 8.2%, respectively. Among the T-cell lymphomas, T-cell LL, ALCL of T/null-cell type, and other nodal peripheral T-cell lymphomas accounted for 6%, 4.3%, and 2.9% of all cases, respectively.

Flow Cytometric Immunophenotyping (FCI)

FCI is useful in diagnosing and subtyping chronic lymphoproliferative disorders (CLPDs) in peripheral blood, BM, other body fluids, and in FNAC of lymph nodes [13–18]. Most labs do 3–4 color FCI, a few do 6 colors and a selective few do 8–10 color FCI. The data generated by FCM are not limited to the percentage of cells positive with a marker, but extend to simultaneous expression of markers, pattern evaluation, and the intensity of staining. FCI has its own limitations. Evaluation of possible T-cell-rich B-cell lymphoma or a HL can be problematic because of scanty tumor cells and also nodal fibrosis, which might prevent recovery of Reed–Sternberg cells. Similarly, necrotic tumors can give negative results if the small sample used in the flow analysis for FCI does not contain any viable tumor cells.

Most lymphomas are substantially defined by their immunoprofile. When there is a discrepancy between morphology and FCI (technical failure, incorrect diagnosis, or a genuinely aberrant result), further investigations are needed to clarify the results. The final report must highlight discrepancies and should suggest an explanation for abnormal or conflicting immunophenotypic findings.

FCI is the most reliable and robust test for the diagnosis of CLPDs in peripheral blood (Figs. 16.6, 16.7, 16.8 and Tables 16.10 and 16.11). It is performed virtually in all cases with lymphocytosis to confirm the diagnosis (suspected by PBS morphology) and to further subtype the disorders [5]. Mostly these are B-cell phenotype; common subtypes include chronic lymphocytic leukemia followed by follicular lymphoma, mantle cell lymphoma, hairy cell leukemia, Waldenstrom macroglobulinemia, splenic marginal zone lymphoma, and the rarer T-cell CLPDs which may include T/NK large granular cell leukemia, T-cell prolymphocytic leukemia, Sézary syndrome, adult T-cell leukemia/lymphoma, etc. Thus, immunological markers will allow the separation of B- from T-cell-derived diseases and, within the B-cell conditions, will establish the clonal nature of the lymphocytes by showing Ig light chain restriction and subtype CLPDs based on morphology and immunoprofile. Documentation of B-cell clonality (by doing light chain restriction) in cases with borderline lymphocytosis is important to differentiate neoplastic, clonal B-cell disorders from benign conditions. It is important to note that clonality is not equivalent to malignancy. Multicolor nature of FCI helps do an extensive panel B/T/NK/plasma cell marker to reach a diagnosis. FCI of mature B-cell non-Hodgkin lymphoma involving the bone marrow and peripheral blood by flow cytometry showed CLL (68.5%) is the commonest followed by follicular lymphoma (30 cases, 8.5%), mantle cell lymphoma (20 cases, 5.5%), splenic marginal zone lymphoma (18 cases, 5%), hairy cell leukemia (18 cases, 5%) [15].

FCI can also be performed on body fluids (like pleural fluid, ascitic fluid, and CSF) and lymph node aspirate samples with low cell yield. Being a rapid diagnostic method, it is helpful in the diagnosis of oncological emergencies like superior vena cava syndrome (SVCS). FCI performed on the lymph node aspirate may solve the diagnostic dilemma especially when poor fixation or processing of the tissue hampers the interpretation of IHC markers. These two techniques of biopsy with IHC and aspirate with FCI are complementary.

Lymphoma Diagnostic Panels

Bone marrow aspirate with subtle involvement is difficult to comment for involvement by NHL. Usually a cutoff of 30% lymphocytes or 5% of large atypical cells on differential count is considered suspicious of involvement. However, FCI

may be required to confirm the nature of lymphoid cells. The focal clustering on imprint smear gives better idea of marrow involvement. Circulating lymphoma cells in peripheral blood smear (PBS) are seen in 40–50% of cases with bone marrow involvement. Incidence of circulating lymphoma cells is highest for low-grade lymphomas like CLL, MCL, FL, SMZL, HCL, ATLL, mycosis fungoides, etc. Cytological features of lymphoma cells are best appreciated on PBS. FCI (combination of various antibodies) assists in the diagnosis (determining the clonality of B/plasma cells) and classification of B/T/NK/plasma cell lymphoid neoplasm. Also, it gives simultaneous co-expression of various B/T/NK/plasma cell markers and even aberrant expression on these cells. Also, it can provide additional prognostic information such as CD38 and ZAP-70 expression in CLL. With increasing sensitivity and specificity, it is becoming an established method for the evaluation of minimal residual disease. Correlation of aspirate and trephine findings are mandatory.

Approach of Lymphoma Diagnosis on Flow Cytometry

Approximately 95% of low-grade lymphomas presenting as leukemia are of B-cell phenotype [15]. The commonest approach in mature B-cell lymphoproliferative disorders is shown below (Figs. 16.6, 16.7, 16.8 and Table 16.10) like CD11c, CD25, CD103, and CD123 may be employed (along with B-RAF mutation studies). SMZL becomes diagnosis of exclusion (IgM+, IgD+).

16.4.6.3 Cytogenetics in Hematolymphoid Neoplasms

Cytogenetics has an important diagnostic and prognostic role in various lymphomas. Interphase fluorescent in situ hybridization (FISH) is mainly performed in lymphomas as it is difficult to get adequate mitoses in lymphoma cells. FISH analysis can be performed on peripheral blood, bone marrow aspirate, fluids or cytology smears, lymph node paraffin sections, etc. The panel for FISH is mainly dependent on the final tissue diagnosis. In B-cell lymphoma, IgH gene rearrangement and TCR gene rearrangement by FISH do the screening role to define the presence of lymphoma or not. Specific chromosome abnormalities are strongly associated with particular subtypes, for example, t(8;14) or variants in Burkitt lymphoma and t(11;14) in Mantle cell lymphoma. Refer to Table 16.9 for common cytogenetic abnormalities seen in HLN.

16.4.6.4 Diagnostic Panels for Molecular Tests

T and B lymphoid cells are involved in immune function for the recognition of foreign antigens by the specificity of their surface T-cell receptor (TCR) and immunoglobulin, produced by gene rearrangement. Each T and B lymphocyte carries a unique arrangement of TCR or immunoglobulin genes, which helps in the identification of clonality of mature T- and B-cell proliferations. IGH and TCR gene rearrangement by polymerase chain reaction (PCR) can be performed on any sample, that is, peripheral blood, bone marrow aspirate, or paraffin-embedded tissues (lymph node or bone marrow biopsy). Molecular studies are important for diagnosis as well as detection of minimal residual disease. While the use of PCR for specific translocations such as t(14:18), and for detection of T-cell and B-cell clonality based on TCR and Ig heavy and light chain gene rearrangement studies, is an important diagnostic test, false positives and negatives are not uncommon; therefore, PCR results should not form the sole basis for diagnosis.

Conventional karyotyping and FISH for specific translocations are more useful at the time of diagnosis than during disease monitoring, as they are sensitive in lymph node/spleen and relatively insensitive in blood and bone marrow. Molecular monitoring of a known detectable translocation is much more sensitive, although FISH will pick up a higher proportion of cases with standard translocations at diagnosis.

Take Home Message: Lymph Node/ Extranodal Sites

1. Avoid making a definitive diagnosis on FNAC except in suspected cases of relapse of HLN. FNAC may be performed to obtain tissue for flow and molecular studies.

2. Avoid frozen section if tissue is scanty. It may lead to freezing artifacts and depletion of the tissue.
3. Biopsy is mandatory (largest palpable node, needle core). Pediatric surgeon should preferably do a pediatric node biopsy.
4. Quality fixation and processing are extremely important so as to obtain a good quality H&E section (2–3 μm thick).
5. An accurate diagnosis of HLN requires multidisciplinary approach which includes clinical, biochemical, radiological, hematological parameters with morphological and immunophenotyping analysis by IHC and/or FCI.
6. Clinical history is extremely important including age of the patient, complete blood counts, peripheral blood smear, and BM aspirate findings.
7. Spend adequate time on scanner/low magnification as it gives vital information about the architecture and contents of the node/tissue biopsy, cellularity, fibrosis, monomorphous/polymorphous nature of the lesion, focal involvement, any abnormal infiltrates, etc.
8. Higher magnification addresses the cytological features in detail.
9. Common IHC markers include CD20, CD3, CD10, MIB-1, and CD23. These will briefly outline the architecture of the node. CD23, CD21, and CD35 are markers for follicular dendritic cells. FDCs are naked looking cells and may rarely be binucleate.
10. Lymphoma may show diffuse, nodular, or mixed pattern of involvement. It may even show sinusoidal (ALCL, HSGDTCL, MZL) or an Indian file pattern (EMMT).
11. Scanty CD4+ Th cells may be seen within the germinal center. These are cells of origin of AITL. Rarely, they are increased and may be confused with follicular lymphoma as they express CD10 and bcl2.
12. Sheets of CD20+ cells favor a lymphoma; however, this needs to be substantiated with other factors.
13. Mimics of lymphomas include viral infections, granulomatous infections, drugs (phenytoin, etc.), and autoimmune diseases. Other common mimics include Kikuchi Fujimoto disease, Kawasaki disease, Castleman disease, Kimura disease, and toxoplasmosis. Toxoplasmosis classically involves posterior cervical nodes and shows a triad of microgranulomas, follicular hyperplasia, and monocytoid B-cell hyperplasia. Kikuchi Fujimoto disease classically involves young Asian women, shows geographical necrosis on low power and abundant karyorrhectic debris on high power. Burkitt lymphoma shows highest number of apoptotic bodies and karyorrhectic debris. Viral infections might show increase in CD20 and CD30+ immunoblasts.
14. Thoracic nodes might show abundant anthracotic pigment, and pelvic nodes show hyalinization.
15. Infidelity of IHC stains is well known. CyclinD1 may also be expressed in a plasma cell neoplasm and hairy cell leukemia, in addition to mantle cell lymphoma. CyclinD1-negative MCL are also known. Sox11 is another marker for MCL. Bcl2 stain has a limited role and is primarily used in differentiating BL from DLBCL.
16. Follicular lymphoma grade 1 is a common differential diagnosis of MCL, while FL grade 3 is a differential diagnosis of follicular hyperplasia.
17. A lab must possess adequate IHC panels (including those for non-hematolymphoid tumors, like Pan-cytokeratin, S-100, round cell tumor panel, etc.).
18. Diagnosis is a multidisciplinary approach and includes clinical, morphological, phenotypical, and molecular data so as to categorize lymphomas into germinal center (GC) and extracentric subgroups. GC entities include NLPHL, follicular, BL, AITL, and DLBCL with (GC profile), and they express bcl6, CD10, and/or the GC-homing chemokine CXCL13. Post-GC entities like classical HL, MZL, and LPLs, half of CLL/small lymphocytic lymphoma, DLBCL with "activated" or post-GC profile, primary effusion lymphoma, plasmacytoma, and myeloma express MUM.1 and/or CD138, harbor static

rather than ongoing SHM, and may harbor EBV in higher latency states.
19. Cotswold Modification of Ann Arbor Staging System is used in staging of lymphomas.

16.5 Bone Marrow Biopsy Interpretation

16.5.1 Introduction

Bone marrow (BM) is encased by cortical bone and traversed by trabecular bone and consists of hematopoietic cells, capillary venous sinuses (basic structural unit of BM), and extracellular matrix within the bony trabeculae. Microenvironment consists of stromal cells consisting of fibroblasts, macrophages, adipocytes, and endothelial cells. Extracellular matrix consists of collagen, fibronectin, laminin, thrombospondin, and proteoglycans. Fat cells are admixed with hematopoietic cells. Approximately 0.01–0.05% of BM mononuclear cells are hematopoietic stem cells [19, 20]. Major hematopoietic cells belong to any of the three lineages, namely myeloid/monocytic, erythroid and megakaryocytic apart from lymphocyte, natural killer cells, and plasma cells. Myeloid/monocytic lineage includes neutrophils, eosinophils, basophils, monocytes/macrophages, dendritic cells, and mast cells (and their precursors).

BM biopsy reporting has been a difficult area for pathologists for various reasons. Most importantly, there is lack of training and experience in hematopathology, followed by suboptimal tissue processing protocols and unavailability of immunohistochemistry (IHC) stains. Few centers in India do trephine biopsy on a regular basis. Trephine biopsy specimen is immediately transferred into a suitable fixative and decalcified, and further embedded in paraffin wax [21]. Section is stained with hematoxylin and eosin (H&E), with or without a reticulin, iron, and Giemsa stain. Need for other histochemical stains (like AFB, etc.) or IHC is determined by the clinical and the morphological features. BM biopsy reporting is done in a systematic manner, starting from structure of bone (cortical as well as trabecular) to various marrow elements (which include the vessels, stroma, and the hematopoietic or other tissue).

Aspirate versus biopsy: BM aspirate and biopsy are complementary and have their own advantages. Though morphology is best studied in an aspirate smear stained by a Romanowsky stain, trephine in addition helps in the assessment of bony structure, architecture, cellularity, patterns, and deposits, if any. The combined information becomes critical in management of many cases.

16.5.2 Indications for a Trephine Biopsy

1. Investigation of a peripheral blood abnormality if cause cannot be ascertained by other means.
2. Pyrexia of unknown origin for granulomatous disease or parasites like leishmaniasis, etc.
3. Assessment of cellularity as in aplastic anemia.
4. Inadequate or failed aspirate/dry tap as in hairy cell leukemia.
5. Suspected bone marrow fibrosis (myelofibrosis).
6. Staging purposes as in lymphomas and round cell tumors.
7. Suspected focal lesions/nodular deposits (as in suspected granulomatous disease, myeloma, Hodgkin or non-Hodgkin lymphoma).
8. Unexplained pancytopenia and leucoerythroblastic picture.
9. Diagnosis of hematolymphoid neoplasm (HLN) especially in myelodysplastic syndrome (MDS).
10. Study BM architecture or bone structure.
11. IHC in cases of minimal residual disease.

Site and technique of biopsy: Patients are informed about the procedure in advance and a written consent is obtained if the procedure is to be carried out under general anesthesia (consent taken from parent/guardian in pediatric patients).

Verbal consent is usually considered sufficient if the patient will be fully conscious during the biopsy; however, local hospital/laboratory policy should be followed in this regard. Thrombocytopenia is, as such, not a contraindication for doing a trephine biopsy. Adequate clinical data, peripheral blood smear, CBC findings, and any other necessary details must accompany the BM biopsy requisition form. It is a painful procedure and should be performed only when there is a clear-cut clinical indication and should be done under adequate local anesthesia in adults and a suitable anesthesia in pediatric patients. A trephine biopsy is usually most easily carried out on the posterior superior iliac spine, with the patient in the left or right lateral position and with the knees drawn up. Adequate local anesthesia must be given with particular attention being paid to infiltrating an adequate area of the periosteum. The biopsy needle should be firmly fixed in the cortex of the bone before the trocar is removed, to avoid including extraneous tissues in the biopsy specimen. Only appropriately trained personnel, usually consultant hematologist, hematopathologist, or a trainee in these disciplines should carry out trephine biopsies. It is preferable to use disposable needles to avoid the risks associated with cleaning reusable needles including Jamshidi and Islam needles. Do not use blunt needles so as to avoid crushing artifacts. A trephine biopsy and aspiration biopsy can be carried out through the same skin incision but different tracts to avoid a hemorrhagic BM trephine, a common artifact [19]. All biopsy samples are considered a potential biohazard. Thus, a possibility of tuberculosis, HIV, or hepatitis B needs to be carefully considered in each case. If tuberculosis is suspected, a fresh sample should be sent directly to the microbiology laboratory. Tissues may be collected upfront in different fixatives for ancillary studies including DNA/RNA and flow cytometry.

BM aspirates as well as touch imprint smears should be prepared immediately, using wedge slide method (similar to peripheral blood smear preparation). Imprint smears should be prepared by rolling of trephine biopsy core on glass slides. Imprint smears from the biopsy are also made, and these are more important in cases where aspiration is difficult (dry tap, fibrotic BM). Imprint smears give invaluable information in addition to BM aspirate smears, as seen in hairy cell leukemia. If trephine sample is clotted, the sample may be fixed (formalin) and sent for histopathological examination. Trephine biopsy should be immediately transferred to appropriate fixative. Keep fixative away from the aspirate smears to avoid staining artifacts.

Adequacy of biopsy: Biopsy size after processing should be at least 1.6 cm, and the biopsy on microscopy should show to contain at least five to six intertrabecular spaces. Amount of assessable marrow elements within the trephine is of more importance than the total length of the biopsy [19, 20, 22]. A pediatrician should be aware of the fact that BM biopsy in a child might contain a majority component of cartilage limiting interpretations owing to inadequacy of marrow component. Cartilage can be grossly identified as a glistening blue core. Single BM biopsy is enough for obvious reasons. A higher pickup rate seen in bilateral biopsies done in staging purposes do not really affect the disease management.

Processing of biopsy specimens and staining of sections: After making touch imprints, the biopsy specimen is subsequently put in a suitable fixative and later decalcified and processed further. There are many protocols for BM processing. We follow Hammersmith protocol [21], which recommends aceto-zinc formalin (AZF) as a fixative. Addition of zinc, to stabilize nucleic acids, can protect against hydrolysis in the presence of a weak acid and hence AZF preparations can effectively speed up decalcification without detriment to morphology or antigen preservation. Later, tissue is transferred to a decalcification solution (10% FA and 5% formaldehyde). The process of fixation and decalcification takes less than a day. Plastic embedding of trephine biopsy specimens is practiced at very few centers. Its main advantages include obtaining very thin sections, which stain well with Giemsa leading to good morphological details. It also obviates the need of any decalcification. However, it is a cumbersome, technically demanding, and expensive procedure. Moreover, good quality 2–3 μm thin

sections can be obtained from paraffin wax-embedded biopsy specimens; hence, plastic embedding of BM biopsy has lost its popularity. All biopsy specimens should have sections stained with hematoxylin and eosin (H&E). Stains like reticulin, iron, Giemsa may be performed as they might provide additional valuable information. Giemsa stain also helps in highlighting mast cells. An iron stain is unreliable as it may be lost during decalcification and, however, may be useful in few cases. Most laboratories cut a single section (H&E stain), while others obtain 2–3 or more deeper sections at different levels in each case. Few centers do iron and reticulocyte stains as a routine in every case. Special stain for microorganisms (AFB, GMS, PAS) and IHC should be applied as and when required.

16.5.3 Different Samples and Ancillary Techniques Required

16.5.3.1 Peripheral Blood and BM Aspirate Specimens

Apart from morphological assessment after Romanowsky staining, it is used for cytochemistry, FCM, PCR, FISH, and conventional cytogenetic studies. It should be collected in an appropriate anticoagulant (EDTA, etc.), and the sample is best studied within 24 h of collection. Heparin is preferred if a delay is anticipated (up to 72 h).

Flow Cytometry: Samples for analysis by FCM should be kept at room temperature and received within 24 h of collection, as there can be sample degeneration, reduction in antigen strength, or complete loss of antigen with time (maximum within 2 days of collection). *Cytogenetics*: Heparin is the preferred anticoagulant for peripheral blood and BM. Unfixed blood films made using silane-coated slides can be used for FISH. Such films must be made fresh, but can give adequate results even after years if stored at room temperature. They have the advantage that morphology can be seen alongside FISH. *Molecular Tests:* EDTA may be used as an anticoagulant for molecular tests such as RT-PCR. A sample of fresh tissue may be rapidly frozen and stored for subsequent analysis such as DNA analysis, if required.

16.5.3.2 BM Trephine Biopsy: Collection and Preparation

Lab needs to follow standard published protocols for processing of BM biopsy specimen [21]. Fixatives should be available in the BM operation theaters. *Morphology*: Obtain good quality thin sections (<3 μm thick). BM trephine sections are stained with hematoxylin and eosin. Additional stains include reticulin, Giemsa, and an iron stain. Each slide should be examined initially for cellularity and trilineage hematopoiesis. Any abnormal infiltrate should be identified and described in terms of cellular morphology and type/pattern of infiltration. Lymphoma infiltration can be in the form of one or a combination of the four categories including interstitial, paratrabecular, nodular, and diffuse pattern. Subtle BM infiltrates may be seen in ALCL, SMZL (intrasinusoidal infiltrates), etc. and are picked up by IHC.

16.5.3.3 Body Fluid Samples

Body fluids like CSF and pleural fluid are best examined within 4–6 h of collection. As quantity of CSF is small, selection of IPT panel becomes crucial so as not to miss the cells of interest. Pleural fluids in adolescents are generally involved by T-lymphoblastic lymphomas, so it is important to accordingly do cytoplasmic CD3 and Tdt apart from routine stains. Similarly, ascitic fluid may show the presence of Burkitt lymphoma or DLBCL. Cytomorphology along with FCI is helpful in such situations.

16.5.4 BM Biopsy (Trephine)

BM biopsy (trephine) are studied under the following subheadings.

16.5.4.1 Morphological Approach to a Normal BM

Important components are quality of processing, adequacy of BM, bony architecture, architecture

and cellularity of BM, trilineage hematopoiesis, any abnormal cells or aggregates, and background stroma. H&E-stained sections are best examined systematically at scanner view (1X or 2X objective), followed by a low-power (10X) and high-power (40X objective) examination. Oil immersion examination is best done for peripheral blood and BM aspirate smears (100X). Scanner/low-power examination is important for the evaluation of the adequacy of the biopsy specimen, assessment of cellularity (uniform or patchy), patent sinuses, adequacy of megakaryocytes, and detection of focal lesions or any deposits. It also helps in selecting areas for further high-power evaluation. Any abnormality in the trabecular bone is also best assessed in scanner/low power. Focal lesions that may be noted at low power include granulomas and focal infiltrates of mast cells, lymphoma cells, or other tumors.

Trephine is the best modality to judge BM cellularity, done at the scanner view. Cellularity is based on the proportion of hematopoietic cells and adipocytes (fat cells). Cellularity is maximum in neonates and in young children (100% hematopoietic cells and negligible fat cells) and declines with age (Table 16.13) [19, 20, 22]. An arbitrary rule of 100 minus age gives a rough estimate of cellularity. Infants have packed cellularity, and this should not be called as a hypercellular marrow and appears blue on morphology as it has an increased number of lymphoid cells and hematogones [19, 20, 22]. Likewise, old age individuals will reveal a less cellular marrow with increased adipocytes and lesser hematopoietic cells, normal for their age. It is difficult to comment on cellularity, if the biopsy is small, hemorrhagic, or contains subcortical bone only. However, in our experience, a significant number of trephine done in infants as in staging of round cell tumors reveal cellularity less than what is defined in literature. A tiny biopsy containing only subcortical bone marrow may be inadequate for assessment because it normally shows hypocellular marrow and may mislead a pathologist in overdiagnosing hypocellular BM. It is difficult to comment on cellularity of BM in a fibrotic marrow, and also in edematous stromal background, which may be due to treatment-related changes. There are various systems of grading of fibrosis and popularly followed is the European consensus [23] as shown in Table 16.14.

At low and high power, the location of cells of erythroid and granulocytic lineages and their relative proportions can be assessed, the nature of any focal lesions can be determined, and sinuses/blood vessels are examined. Examination at high power is important for finer morphological details. Dyspoiesis and other finer details including microorganisms and parasites (cryptococci, histoplasmosis, leishmania donovani bodies) are best detected in high power/oil immersion (these are best appreciated in aspirate). After examination of the H&E-stained sections, a reticulin and iron stain may be examined. The reticulin should be graded, according to a standardized published system, and any focal increase in reticulin deposition should be noted (Table 16.14). The presence of focal reticulin deposition is an indication to re-examine the H&E-stained sections to exclude any possibility of a focal infiltrate like lymphoma or mast cell lesion or a granuloma. This may demand deeper sections of trephine biopsy. Specialized cytochemical stains (such as Congo red stain or Ziehl–Neelsen stain) or IHC may be performed as and when needed.

Trilineage Hematopoiesis and Other Cells

Hematopoiesis is best studied on low- and high-power examination. It is the next most important feature (after cellularity) where pathologist

Table 16.13 Age related BM cellularity

Age	% Cellularity	% Granulocytes	% Erythroid	% Lymphocytes
Newborn	80–100	50	40	10
1–3 months	80–100	50–60	5–10	30–50
Child	60–80	50–60	20	20–30
Adult	40–70	50–70	20–25	10–15
Old age >70 years	20–40	50–70	20–25	10–20

Table 16.14 European consensus on the grading of bone marrow fibrosis [23]

Grade 0: Scattered linear reticulin with no intersection (crossovers) corresponding to normal bone marrow
Grade 1: Loose network of reticulin with many intersections, especially in perivascular areas
Grade 2: Diffuse and dense increase in reticulin with extensive intersections, occasionally with only focal bundles of collagen and/or focal osteosclerosis
Grade 3: Diffuse and dense increase in reticulin with extensive intersections with coarse bundles of collagen, often associated with significant osteosclerosis

Table 16.15 Normal adult values for BM differential cell counts

Cell type	Normal range (%)
Myeloblasts	0–3
Promyelocytes	2–8
Myelocytes	10–13
Metamyelocytes	10–15
Neutrophils plus band	25–40
Eosinophils and precursors	1–3
Basophils and precursors	0–1
Monocytes	0–1
Erythroblasts (early)	0–2
Other erythroid elements (late)	15–25
Lymphocytes	10–15
Plasma cells	0–1
Megakaryocytes	1%
Mast cell	0–1

should look for trilineage hematopoiesis (myeloid, erythroid, and megakaryocytic lineages), in amount, proportions, and morphology and other cells including lymphoid cells (Table 16.15). Lymphocytes may mimic erythroid precursors and it can be tricky to differentiate them.

1. *Erythroid series cells*:

   Normal M:E ratio is 2:1 to 4:1. Erythroid cells may occur singly interspersed with myeloid cells or else come in clusters/colonies. They have a uniformly round nuclear contour, prominent cell membranes, perinuclear halo, and a dense homogeneous chromatin (ink dot). The nucleus appears very dark, and chromatin character is difficult to ascertain. Erythroid colonies are small and intertrabecular in location and are commonly seen in anemia, myelodysplastic syndrome (MDS), and regenerating BM post induction. Megaloblasts are larger round to polygonal cells and have moderate cytoplasm, round nuclei with vesicular chromatin, and a crisp nuclear membrane with 2–3 nucleoli. CD36, CD235, and CD71 highlight erythroid cells. C-kit (CD117) may weakly highlight both megaloblasts and myeloblasts and, however, is strongly expressed in mast cells.
2. *Myeloid series cells*:

   It is the most prominent series in an adult BM and accounts for 50–70% of all nucleated cells. These are granular cells with pinkish granular cytoplasm. Granules of mast cells and basophils get washed off and are not seen on H&E stain. The most immature myeloid cells are myeloblasts and promyelocytes, which are located in paratrabecular and perivascular location, while mature cells like neutrophils and metamyelocytes are located intertrabecularly. These might be confused with megaloblasts and histiocytes (agranular). MPO highlights myeloid series cells on IHC. Myeloid proliferation is seen in infections and also in neoplasms, like myeloid neoplasms, etc. Promyelocytes are large-sized myeloid precursors and have pinkish granular cytoplasm.
3. *Megakaryocytic series cells*:

   Megakaryocytes are largest hematopoietic cells, smaller only to fat cells in BM trephine, and are easily picked up on the scanner view. They constitute less than 1% of all nucleated BM cells. BM biopsy is the best mode of examining megakaryocytes in number, morphology, and distribution. They are distributed all over, mostly adjacent to sinusoids. There are approximately 2–4 megakaryocytes per high-power field on a trephine. They might show emperipolesis, where normal hematopoietic cells are seen within the cytoplasm of the megakaryocytes. They are multilobated (nuclear lobulations) and have a granular cytoplasm, and should not be confused with multinucleated giant osteoclasts.

   A megakaryocytosis may be congenital or acquired and, however, is extremely rare.

Megakaryocytes may be increased in BM in megakaryocytic thrombocytopenia, lupus-associated thrombocytopenia, AIDS, MDS, chronic myeloproliferative disorders (may be associated with myelofibrosis), etc. [19]. Morphology of megakaryocytes is an important factor in differentiating early premyelofibrosis from essential thrombocytosis. CD36, CD41, and CD61 are stains to highlight megakaryocyte and AML-M7.

4. *Lymphoid series cells and lymphoid aggregates*:

Larger number of lymphoid cells is seen in pediatric BM, where they may reach up to 60% of all nucleated BM cells. Morphologically, these are singly scattered, darkly stained, small-sized cells. They might have angulated nuclear contours with appreciable chromatin (in comparison with erythroid cells which have a black round dot like nucleus with a clear halo). Hematogones and benign lymphoid aggregates are seen in benign reactive conditions and need a special mention.

(a) *Hematogones*: BM may show immature lymphoid precursors called hematogones, seen normally in larger numbers in normal infant/pediatric BM. They are increased following viral infections, post chemotherapy, post-transplant, and in association with round cell tumors, lymphomas, etc. On morphology, they are small to medium sized, with scant cytoplasm, round to irregular nuclei with dense homogeneous bland-looking chromatin, and inconspicuous nucleoli. They appear as diffuse subtle infiltrates, singly scattered cells, and rarely as small aggregates. Hematogones are a spectrum of B cells (CD19+) and express a range of markers with variable intensity depending upon its stage of maturity. CD20 is the strongest in mature hematogones (stage 3) while CD34, CD10, and Tdt are the strongest in immature hematogones (stage 1). Thus, hematogones show a variable expression of CD20, CD10, CD38, CD56, CD34, and Tdt. They are best studied on FCI and have a classical pattern of lymphoid maturation (dot plots of CD20 and CD10, and of CD34 and CD38). They may be confused with lymphoblasts, and few cases continue to be misdiagnosed as B-cell acute lymphoblastic leukemia. Hematogones are important to understand as they mimic tumor cells during minimal residual disease (MRD) detection of B-ALL. In such cases, morphological examination of peripheral blood smear may help as it reveals an otherwise normal differential count with near normal complete blood counts. Hematogones are normally not seen in peripheral blood; however, precursor B cells may be seen in neonates. BM aspirate morphology also reveals trilineage hematopoiesis in cases with increased hematogones.

(b) *Benign lymphoid aggregates*: Lymphocytes appear singly scattered and rarely form lymphoid aggregates. Cytology of lymphoid aggregates is best seen on aspirate while patterns and extent of involvement are best picked up on trephine. Paratrabecular aggregates are those which are adjacent to trabecular bone, almost kissing the bone, while intertrabecular aggregates are within two bony trabeculae, far away from bony surface. Most paratrabecular lymphoid aggregates are neoplastic, commonly seen in follicular lymphomas (and mantle cell lymphoma). Thus, benign lymphoid aggregates are usually small, few in number, perivascular, and commonly intertrabecular in location. They are mostly well circumscribed (except in HIV patients where they may be poorly demarcated). These aggregates are polymorphous and composed of mature lymphoid cells may also include plasma cells, histiocytes, and mast cells and occasionally show germinal centers. These are commonly seen in old age, autoimmune disease, and MDS. IHC may be helpful in differentiating benign from malignant lymphoid aggregates. On IHC, most of the lymphoid

cells are CD3+ T cells admixed with a few CD20+ B cells. Other important markers include CD10, CD5, CD23, and cyclinD1. Look for RS or RS-like cells and appropriate IHC (CD15, CD30, etc.) may be done in a known case of HL to confirm infiltration. To rule out a T-cell lymphoma infiltration, an extensive T-cell panel is required including CD2, CD3, CD4, CD5, CD7, and CD8, along with CD10, Tdt, CD56, and CD30. T-cell clonality is best studied by T-cell receptor gene rearrangement studies by polymerase chain reaction.

5. *Plasma cells*:

   Plasma cells constitute less than 1% of all nucleated BM cells. They may be increased in neoplastic conditions like plasma cell neoplasm, Hodgkin lymphoma, peripheral T-cell lymphoma, AITL, etc. and also in infectious diseases like leishmaniasis, tuberculosis, HIV, etc. Plasma cells may be seen scattered singly or in clusters, nodules, and sheets, as seen in plasma cell neoplasm. They are medium to large sized, egg-shaped cells with abundant blue cytoplasm, and a round eccentric nucleus, which may show a cartwheel nuclear pattern. Plasma cells and lymphocytes are the only remaining hematopoietic cells in the BM of patients of aplastic anemia where rest of the normal hematopoietic cells including megakaryocytes is depleted. These cases have been sometimes mislabeled as plasma cell neoplasm. Plasma cells are highlighted with CD138 (and Mum1), and their clonality is demonstrated by kappa and lambda light chain restriction studied by IHC and FCM (along with loss of CD19 and/or gain of CD56 and cyclinD1) and gain of CD28 and loss of CD27 and CD81.

6. *Other cells* include mast cells, histiocytes, stromal cells, fat cells, osteoblasts, osteoclasts, etc. Mast cells are best appreciated on aspirate smears (Romanowsky stained). They are singly scattered cells in trephine and have a nucleus like a lymphocyte with moderately abundant cytoplasm, seen adjacent to blood vessels, and express tryptase and CD117. Abnormal mast cells are spindle shaped, come in clusters, may look like focal fibrosis, and express CD2 and CD25. Macrophages and hemophagocytosis are best appreciated on aspirate smears. IHC stains for CD68 highlight histiocytes. Scattered or clusters of histiocytes may also be seen in other disorders as histiocytic sarcomas, Langerhans cell histiocytosis, hemophagocytic syndrome, Gaucher disease, Niemann–Pick disease, etc. Microorganisms like LD bodies, *histoplasma capsulatum* can be identified on high-power examination. Granulomas can be identified on trephine only, not on aspirate. AFB, GMS, and PAS stains are performed for mycobacteria and fungus.

Bone and BM Stroma: Any structural abnormalities in the trabecular bone may be observed at scanner power. BM stroma is very inconspicuous in a normal marrow. Reticulin in a normal BM is very thin and can be seen only with special reticulin stain. Stroma in post-chemotherapy BM might show edema, microvascular adipocytes, etc. in a marrow with variable cellularity (normo to hypo to hypercellular marrow). It is also prominent in chronic myeloid disorders like CML, myelofibrosis, and in rare conditions like serous atrophy. Blood vessels may be highlighted by CD34 stains (stain for endothelial cells and blasts). The eosinophilic trabecular bone is thicker in children and thinner in adults and contains osteocytes. Osteoclasts and osteoblasts are more prominent in children.

Common BM Trephine Artifacts: Poor-quality techniques of BM processing produce many artifacts, thereby limiting morphological interpretations. These may include inadequate or excessive fixation, excessive decalcification, etc. These are the most common causes of error in BM biopsy interpretation. Use of blunt needles might cause crushing artifacts. Hemorrhagic BM biopsy is another major artifact caused by aspiration of the marrow from the same site where the biopsy is taken. Other sampling artifacts include a falsely hypocellular BM as seen in subcortical BM, which are normally hypocellular and may be misinterpreted as an aplastic marrow (more so in old age).

BM Infiltration

1. *BM Biopsy and Lymphoma Infiltration*: There are certain histological features which help in differentiating benign from malignant lymphoid aggregates. Features favoring malignancy include topography, that is, localization of the lymphoid aggregates within the bone marrow space (paratrabecular aggregates are highly abnormal), relation to the surrounding tissue (margination or interstitial spillage of lymphoid cells), and increase in reticulin fibers [1]. Lymphomas are best diagnosed by a lymph node (LN) biopsy, with morphology substantiated with various ancillary techniques, mainly IHC. However, there are lymphomas, which initially present as lymphocytosis (chronic leukemias) and fall in a broad category of chronic lymphoproliferative disorders (CLPDs). Morphologically, these are more like mature lymphoid cells. CLPDs are best diagnosed on peripheral blood smear examination and immunophenotyping by FCM. BM biopsy in lymphomas is done mainly for staging purposes, except in few cases where it may help in diagnosis, as in hairy cell leukemia (HCL) which commonly presents as pancytopenia and reveals a dry bone marrow tap due to myelofibrosis. HCL reveals interstitial to diffuse interstitial increase in atypical lymphoid cells (substantiated with IHC like CD20, CD11c, CD25, CD103, CD123, etc.). BM biopsy is also helpful in follow-up of lymphoma cases to detect residual focal disease when a BM aspirate is normal. HL is difficult to diagnose on aspirate and, however, can easily be detected on trephine biopsy histology where classical RS cells, mononuclear RS-like cells, fibrosis, granulomas, etc. may be seen. CD20 also highlights small clusters of intrasinusoidal infiltration in BM as seen in SMZL. CD3 will also pick up intrasinusoidal spread of tumor T cells in cases of hepatosplenic gamma-delta T-cell lymphoma (which otherwise might be missed on H&E stain).

   Trephine and aspirate are complementary techniques in lymphoma evaluation because either may reveal infiltration when the alternative procedure fails to do so. A trephine biopsy performed for staging sometimes shows discordant morphology (grade) where a nodal high-grade large cell lymphoma might look like a low-grade small cell lymphoma on BM biopsy.

   BM biopsy in cases of lymphoma might show various patterns of infiltration. It may show diffuse involvement as seen in chronic lymphocytic leukemia (CLL) and patchy or partial involvement as seen in HL. IHC has an important role mainly in the detection of lymphoma involvement by singly scattered lymphoma cells, as in ALCL. Common patterns of marrow involvement in trephine biopsy in lymphoma are:

   (a) *Diffuse pattern*: Infiltrates that efface marrow architecture, for example, ALL and CLL. Hairy cell leukemia (HCL) has a diffuse or interstitial involvement.
   (b) *Interstitial pattern*: Tumor cells infiltrate the BM without disruption of overall marrow architecture, commonest example being hairy cell leukemia (HCL) and others like BL, ALL, PTCL, etc. Normal hematopoietic cells are intermingled with the tumor cells.
   (c) *Paratrabecular pattern*: Lymphoid cell aggregates extend along the bony trabeculae, internally along the bony surface and extend away from trabeculae. It might show a rough wedge-shaped infiltrate (with broad base toward the trabecular bone) or a wavy infiltrate kissing the trabecular bone. It is classically seen in FL, and also in MCL, splenic marginal zone lymphoma (SMZL), and PTCL. Poorly delineated paratrabecular aggregates may be seen in T-cell-rich B-cell lymphomas.
   (d) *Intertrabecular pattern*: This may be focal and random. It is a common pattern seen in benign lymphoid aggregates, may come as nodular form, and is commonly seen in SMZL, CLL, and MCL. Patchy intertrabecular infiltrates with fibrosis and polymorphous background along with RS-like cells are commonly seen in HL. Patchy or nodular deposits may be

seen in myeloma. Patchy, poorly circumscribed lymphoid infiltrates may be seen in AITL, with fibrosis and vascular proliferation in the background (morphological features similar to those in lymph node).

(e) *Sinusoidal or intravascular*: Commonly seen in T-cell lymphomas like ALCL and hepatosplenic T-cell lymphoma (HSTCL) and B-cell lymphomas like SMZL and intravascular lymphoma and also seen in deposits of metastatic carcinomas or a round cell tumor.

HL may have different patterns, patchy focal involvement to extensive sclerotic involvement. It may consist of polymorphous cellular populations of lymphocytes, eosinophils, plasma cells, histiocytes, neutrophils with/without RS-like cells (or mononuclear RS-like cells) in a fibrotic background and may be associated with granulomas and focal necrosis. IHC stains (LCA, CD3, CD20, Pax5, CD15, CD30) are helpful. Megakaryocytes may mimic RS cells. Morphologic features of PTCL and HL may overlap in BM biopsy as RS-like cells may be seen in both and in other conditions such as viral infections, CLL, ALCL, and AITL. BM involvement may be suspected even in the absence of classical RS cells, if characteristic milieu of fibrosis (focal to diffuse) containing eosinophils, histiocytes, plasma cells with mononuclear RS-like cells is noted. CD30 will highlight RS-like cells.

2. *Plasma cell neoplasms*: Myeloma diagnosis requires a multidisciplinary approach, from clinical to laboratory to radiological investigations. It might be difficult to obtain a BM biopsy in myeloma cases, as the bones are brittle, thus highlighting the importance of processing the clot like a trephine. However, it might be essential for diagnosis in some cases. Myeloma can be in the form of nodular deposits in the trephine, apart from other patterns like interstitial, diffuse, or mixed types. Aspirates in such cases might reveal a falsely low count of plasma cells where the plasma cell percentage could vary anywhere from 1% to more than 10%. Thus, nodular deposits, clusters, or sheets of plasma cells in a BM biopsy highly suggest a plasma cell neoplasm. Plasma cells can morphologically mimic any other hematopoietic cell, namely blast, promyelocyte, histiocyte, erythroid cell, lymphocyte, etc. Normal plasma cells tend to arrange in perivascular areas; however, clonal plasma cells infiltrate adipocytes and form clusters, nodules, or sheets. Morphological variants include mature, immature, and plasmablastic subtypes. Plasmablasts have high nucleocytoplasmic ratio, fine chromatin, a prominent nuclei, and blue cytoplasm. Plasma cells get highlighted with CD138. CD138 also helps in quantitation of plasma cells, especially when they are interstitially interspersed. CD138 also stains epithelial cells apart from plasma cells. BM biopsy is also important as a baseline for comparison with repeat (sequential) biopsies during follow-up of myeloma. Immunophenotyping by FCM or IHC on trephine can be useful in establishing the presence of a relatively small plasma cell clone. Clonal plasma cells might express CD56, cyclinD1, CD117, CD28, and loss of expression of CD19 and CD27 (expressed in normal plasma cells). Neuroendocrine carcinoma cells also stain with CD138 and CD56, a common morphological differential of myeloma/plasmacytoma. Additional markers should be done for light chain restriction, etc. BM biopsy is also helpful in detecting small deposits of amyloid in suspected cases of amyloidosis (perivascular deposits of extracellular amorphous eosinophilic material). Congo red stain may be performed to look for apple green birefringence under polarizing microscopy.

Waldenstrom macroglobulinemia may also have similar patterns of involvement as myeloma and shows a mixture of plasma cells, lymphocytes, and hybrid lymphoplasmacytic cells. Plasma cells may also be increased in chronic infections as tuberculosis, leishmaniasis, etc., in other neoplasms as HL, AITL, PTCL, etc., and also in leukemias as AML-M5. BM biopsy in cases of aplastic anemia and post induction BM might show only plasma cells and lymphocytes in an otherwise hypocellular marrow. Cases of aplas-

tic anemia and AITL have been misdiagnosed as plasma cell neoplasm on aspirate smears. Thus, it is important to correlate with cellularity on trephine and with other clinical and radiological findings.

3. *Acute leukemia*: A trephine biopsy is not required for the diagnosis/subtyping of acute leukemia, as long as there are adequate tumor cells in the peripheral blood or a cellular aspirate is available. However, paraffin block is an archival material and additional studies like IHC can be performed for subtyping of HLN even at a later stage. Thus, trephine biopsy becomes extremely important in a country like ours where many a time patients are given blood transfusion and/or steroids/heavy metals at the primary health center after/before a diagnosis of acute leukemia. Such patients when referred to a cancer center have no circulating tumor cells, causing a delay in diagnosis. Stained peripheral blood smears or bone marrow (BM) aspirates, if available, might not help further subtype the leukemia. Paraffin block if available at this stage is an invaluable material. BM biopsies in cases of acute leukemias are hypercellular, except for rare hypoplastic AMLs and hypoplastic MDS. BM is packed with similar looking blasts (CD34, Tdt). Rarely, normal or differentiating cells may be seen in the background as in AML-M2, etc. Blasts vary in size from small to large. Lymphoblasts (CD19, CD79a, CD10, Tdt, CD3) are smaller in size with coarse nuclear chromatin. Myeloblasts (CD34, MPO, c-kit) are larger in size and have distinct cytoplasm and vesicular nuclear chromatin. Monoblasts (CD34, MPO, and CD64) are large, with fine nuclear chromatin, and may have a convoluted nuclear membrane. It is important to rule out associated dysplasia in any of the three lineages. CD19 and CD79a are better stains for B-cell ALL as CD20 might be weakly positive or negative in majority of B-cell ALLs. Plasmacytosis is commonly seen associated with AML-M5 and AML-M2 with t(8;21). CD19 is a more popular stain with FCI. Differential diagnosis of acute leukemia in BM may include hematogones, Ewing sarcoma, rhabdomyosarcoma, neuroblastoma, anaplastic large cell lymphoma, and multiple myeloma.
4. *Chronic myeloid neoplasms including chronic myeloid leukemia (CML)*: BM biopsy is markedly hypercellular in CML and shows myeloid and megakaryocytic preponderance. Blasts are not increased in the chronic phase. There is a relative paucity of erythroid series cells. Fibrosis may be seen in CML and other chronic myeloproliferative disorders and shall be graded and reticulin stain may be used. Clusters of megakaryocytes and micromegakaryocytes may be seen. Stains for CD34 and C-kit may be helpful in highlighting blasts. Cellularity and morphology of megakaryocytes may help in distinguishing essential thrombocytosis from early prefibrotic myelofibrosis. In early/prefibrotic myelofibrosis, it is a hypercellular marrow with a prominent granulocytic and megakaryocytic proliferation with a concomitant reduction of red cell precursors with absence of reticulin MF (MF-0 and MF-1). Abnormalities in the megakaryocytopoietic cell lineage include extensive and dense clustering and translocation of megakaryocytes toward the endosteal borders. There is a high variability in size ranging from small to giant megakaryocytes along with prominent aberrations of the nuclei (marked hypolobulation, condensed chromatin, and irregular foldings creating a bulbous, cloud-like aspect) and marked elevation of the nuclear-cytoplasmic ratio, as well as an increased frequency of bare (denuded) nuclei. While in essential thrombocytosis, it reveals an age-matched cellular BM with a predominant megakaryopoiesis, but without a significant erythroid or neutrophilic myeloproliferation. Here the megakaryocytes reveal a more or less random distribution or very loose groupings within the BM space. Megakaryocytes are large to giant cell forms with extensively folded (hyperlobulated) nuclei, surrounded by

well-differentiated cytoplasm. An increase in reticulin is not compatible with early stages of ET.

5. *Myelodysplastic syndrome (MDS) and megaloblastic anemia*: Diagnosis of MDS requires integration of various peripheral blood and BM parameters along with clinical and cytogenetic findings. MDS has various subtypes. Morphological dysplasia is best appreciated on an aspirate smear. Trephine is important in commenting on architecture, cellularity (hypercellular BM), megakaryocyte morphology including dyspoietic forms like micromegakaryocytes, multinucleated megakaryocytes, and also monolobated megakaryocytes (as seen in 5q- syndrome), presence of megaloblasts, abnormal localization of immature myeloid precursors (ALIP), and CD34+ blasts. Fibrosis may be seen in secondary MDS (post-alkylating agent therapy). Epstein–Barr virus infection might produce morphological changes that might mimic MDS features in BM. Common differential diagnoses of MDS in pediatric BM trephine include chronic viral infections, juvenile rheumatoid arthritis, megaloblastic anemia, heavy metal toxicity, chronic myeloproliferative disorders, acute leukemia, etc. Common differential diagnoses of MDS in adult BM trephine include congenital dyserythropoietic anemia, aplastic anemia, hypocellular AML, chronic viral infections, megaloblastic anemia, heavy metal toxicity, AIDS, autoimmune disorders, chronic myeloproliferative disorders, acute leukemia, etc. Micromegakaryocytes are smaller sized cells, have darkly stained nuclei, and scant cytoplasm (bare nuclei). These are commonly seen singly scattered or in clusters in chronic myeloid leukemia, accelerated phase (CML-AP), and MDS. IHC (CD41, CD61, factor VIIIRA) may be done to highlight megakaryoblast and micromegakaryocytes. Small blast-like cells with one or more distinct nucleoli located away from the endosteal surface (paratrabecular) correspond to immature precursors/blasts. Cases with at least three clusters (groups of three to five immature myeloid precursors) or aggregates (more than five myeloid precursors) distributed in the intertrabecular region are defined as being ALIP positive (abnormal localization of immature myeloid precursors). ALIP islands might express CD34 and CD117. These may also be seen in regenerating BM, post chemotherapy, and in CML. BM is hypercellular in megaloblastic anemia, with erythroid hyperplasia. M:E ratio is reversed. Nucleo-cytoplasmic dissociation is most evident in erythroid lineage. Megaloblasts are large round to polygonal cells with large round nuclei, vesicular chromatin, 1–2 small nucleoli, a crisp nuclear membrane, and moderately abundant basophilic/amphophilic cytoplasm. Megaloblasts may be seen as singly scattered cells or else seen in clusters with a background of erythroid hyperplasia. These features may also be seen in MDS. These may be highlighted with CD71 on IHC. Myeloid series also shows giant forms.
6. *Mast cell disease*: Mast cells are best seen in a Romanowsky stained smear. It may show different patterns of involvement in BM mainly paratrabecular, perivascular, parafollicular, and diffuse. Granules are washed off during processing. Mast cells are oval shaped with abundant clear cytoplasm, no mitotic activity, and are associated with fibrosis and admixed eosinophils, histiocytes, and lymphocytes. They may be associated with other myeloproliferative neoplasms like systemic mastocytosis with associated clonal hematological non-mast cell lineage disease. Mast cell disorders are more commonly seen in the Western population. Mast cells are strongly positive for CD117, while CD2 and CD25 highlight clonal mast cells.
7. *BM biopsy and minimal residual disease (MRD)*: MRD detection is best done by multicolor FCM or molecular methods. Best methods to detect MRD in B-ALL, T-ALL, and AML is by FCI, while in case of CML-CP and APML, it is done by molecular methods. CD34 and CD117 stains may be performed to detect scanty myeloblasts in

the BM biopsy. Hematogones may be confused with blasts in post induction BM of B-cell ALL. Clusters of CD34/Tdt-positive cells herald an early relapse of ALL. Hematogones might express CD20, CD10, and Tdt in different stages of their maturation (as stated earlier). We have seen 10–15% Tdt expressing lymphoid cells in post induction regenerating bone marrows (cases of B-cell ALL). Likewise we have seen up to 20% normal myeloblasts (CD34+) in post induction marrow in a case of AML. FCM is a superior technique to differentiate cancer lymphoblasts from hematogones and also cancer myeloblasts from normal myeloblasts, as it gives more flexibility in the form of multicolor immunophenotyping and patterns analysis, using principles of leukemia-associated immunophenotype and concepts of away from normal. One should avoid commenting on the presence of MRD on trephine interpretations.

8. *BM biopsy and metastatic tumors*: BM biopsy is essential if BM examination is being carried out for metastatic workup. Clusters of megakaryocytes may rarely mimic metastatic carcinoma. Though the aspirate is helpful, BM biopsy has better chances of picking up scanty tumor cells. IHC can also be performed for evaluation of unknown primary (like breast, GIT, lungs, prostate, etc.), where common markers include CK, EMA, CK7, CK20, TTF1, PSA, CDX2, etc. Aspirates might show degenerating tumor cells that can easily be missed; however, BM biopsy reveals viable tumor cells in a desmoplastic background. Trephine and aspiration are complementary investigations because either may show tumor cells when the other procedure fails to do so. In rare instances, round cell tumor deposits are seen as clusters in the aspirate and missed in the trephine. This is likely when the aspirate is done at a different angle from where the trephine is done. BM biopsy is done for staging of round cell tumors such as neuroblastoma, rhabdomyosarcoma, and primitive neuroectodermal tumor. Common IHC markers include Mic2, FLI1, desmin, myogenin, MyoD1, synaptophysin, chromogranin, CD56, etc. (along with hematolymphoid markers). Rarely, round cell tumors may mimic acute leukemia on aspirate smears, and immunophenotyping by FCM reveals negative markers (since all markers used are hematolymphoid specific). Trephine is helpful in these cases where a battery of hematolymphoid as well as non-hematolymphoid markers may be done. Sarcomas, melanomas, and germ cell tumors also rarely metastasize to BM.
9. *BM biopsy and granulomatous diseases*: BM biopsy is a part and parcel of investigation for pyrexia of unknown origin. Other specific indications in this context include pancytopenia, fever, and lymphadenopathy. Common causes of granulomatous diseases include tuberculosis, leishmaniasis, sarcoidosis, cryptococcosis, aspergillosis, and histoplasmosis. Granulomas may be seen in trephine sections (but not in a BM aspirate). BM biopsy has an important role in human immunodeficiency virus-positive patients, where granulomas, necrosis, and stain for AFB and other microorganisms should be done. Granulomas in BM may also be seen in various neoplastic conditions such as HL, PTCL, mycosis fungoides, carcinomas, and MDS. Relevant cultures are to be sent after discussion with the clinicians.
10. *BM biopsy and AIDS*: BM may be near normal or show significant morphological changes. Morphologic features may be divided into specific and nonspecific categories. Nonspecific category includes reactive plasmacytosis, megaloblastosis, multilineage dysplasia, poorly circumscribed lymphoid aggregates, diffuse interstitial histiocytic proliferation, hypo-, hyper- to normocellular BM, fibrosis, etc. Specific categories include infiltration by NHL (plasmablastic lymphomas, BL, HL, etc.), Kaposi sarcoma, and opportunistic infections (bacterial, fungal, or protozoal), absence of storage iron, red cell aplasia, etc.

Other Common Findings

(a) *BM Necrosis*: Causes of BM necrosis vary from neoplastic (ALL, AML, myeloma, HL, NHL, carcinomas, PNET, neuroblastoma, etc.) to non-neoplastic (sickle cell anemia, infections, DIC, acute GVHD, etc.) and to treatment related (e.g., post steroids in ALL, post bone marrow transplantation). It should be differentiated from fibrinoid necrosis or stromal edema as seen post chemotherapy or after bone marrow radiation.

(b) *BM biopsy changes post chemotherapy*: Morphologic features of BM in patients undergoing myeloablative chemotherapy may be immediate effects like cell ablation and late effects like bone marrow regeneration (1–2 weeks after the ablative therapy). Immediate effects include apoptosis, followed by fibrinoid necrosis, stromal edema, prominent and dilated sinuses, increased macrophages, and large number of multiloculated adipocytes. Scanty normal hemopoietic cells may be seen, predominantly comprising of lymphocytes and plasma cells. Bone marrow regeneration occurs 1–2 weeks later and may show the appearance of paratrabecular immature myeloid cells, followed by the appearance of neutrophils and erythroid colonies. Megakaryocytes appear late and come in clusters, which is followed by resolution of fibrinoid necrosis (which may stay for a longer duration in some cases). Intra-nuclear parvovirus inclusions may be evident in large-sized proerythroblasts in post induction chemotherapy patients of ALL. These cells resemble RS-like mononuclear cells. These patients may have a delayed recovery of peripheral blood counts. It is a common practice to confuse interstitial edema (commonly seen in post induction marrows) with marrow fibrosis and one has to be careful.

(c) *BM biopsy in post BM transplantation period*: Earliest features (days 1–14) are of cell death, and include fat necrosis, proteinaceous debris, stromal edema, and negligible normal hematopoietic cells. Regeneration starts from day 7 to 14 post-transplant and reveal non-paratrabecular erythroid colonies, myeloid precursors followed by megakaryocytic clusters. Any presence of blasts post day 7 of transplant indicates the presence of residual disease. At the end of a month (day 28), complete engraftment occurs, that is, all cell lines are engrafted. BM may show variable cellularity. Morphologic features of rejection (post day 28) include declining peripheral blood cell counts and features of BM microenvironment damage like fat necrosis, stromal edema, plasmacytosis, histiocytic proliferations, or lymphocytosis.

(d) *BM post growth factor therapy*: Growth factors like GM-CSF lead to interstitial foci of immature myeloid cells in a hypercellular marrow. Myeloid cells show prominent granules and may mimic acute or chronic myeloid neoplasm, and rarely may look like acute promyelocytic leukemia.

16.5.4.2 Immunohistochemistry

Similar to lymph node biopsy, IHC is an important tool in interpretation of HLN in BM biopsies. Use of FCM on peripheral blood/aspirated marrow cells along with IHC provides complementary information, and the two sets of data should be considered together. If FCM data is available from peripheral blood or aspirated bone marrow, it should be taken into account when assessing the trephine morphology. They can aid selection of appropriate antibodies for IHC and, in some cases, reduce or remove completely the need for complex and expensive IHC assessment, avoiding unnecessary duplication. Panels for markers including non-hematolymphoid tumors have been suggested; however, it is at the discretion of the hematopathologist to decide on IHC panel on a case-by-case basis. Tumors like round cell tumors/carcinomas/melanomas may mimic lymphoma, thus it becomes essential to have corresponding markers for these tumors. Minimal suggested basic panel for any hematolymphoid lesion include three markers, namely LCA (CD45), CD20 (B-cell marker), and CD3 (T-cell marker). For blastic lymphoid cell proliferations, CD79a and CD19 are markers of choice in B-ALL (CD20 may be negative). CD20 is a pre-

dictive marker in B-cell lymphomas, as these patients can be given anti-CD20 targeted therapy (rituximab). Additional panels may be suggested based on morphological impression. Common markers in a case of acute leukemia include CD3, CD7, CD20, CD10, CD56, CD4, CD33, CD41, CD61, Pax5, CD79a, CD19, CD34, Tdt, Ckit, AMPO.

16.5.4.3 Cytogenetics/FISH and Molecular Diagnostics

Unfixed BM aspirate sample is a better sample than extracted nuclei from fixed trephine biopsy specimens for conventional karyotyping and interphase fluorescence in situ hybridization (FISH) analysis. However, with newer protocols, good FISH results can also be obtained from trephine cores (fixed tissues). Thin sections offer an advantage of retained architecture and cytology of cells of interest. Trephine FISH may be critical in demonstration of MYC abnormalities in suspected Burkitt lymphoma and in confirmation of t(11;14) in mantle cell lymphomas. PCR may be done to detect IGH and TCR rearrangements using sections from decalcified BM cores.

Reporting of a trephine biopsy: Trephine report shall contain both gross and microscopic descriptions. It should mention the number of BM fragments and length of the longest fragment. The report may begin with a statement on the adequacy of specimen for diagnosis and should then comment systematically on cellularity, bone structure, trilineage hematopoiesis, M:E ratio, dyspoietic features, and stroma and abnormal infiltrates, if any. It should include a comment on special stains including IHC, if any performed. Clinical, peripheral blood smear, and BM aspirate findings should be correlated before the final report is issued. Ancillary techniques like IHC/FCM, cytogenetics, FISH, and PCR reports should be included after correlation to give a comprehensive report based on the current WHO classification of HLN [2]. The report should finish with a final impression of all findings and its clinical significance. Advice on further workup may be given, if required. Provisional report may be sent if deeper sections or special stains are pending or if a second opinion is being sought. This is followed by a final report. Provisional as well as final report must contain the name and signatures of the authorized signatory or by a secured computer authorization.

Who should report a trephine biopsy? Ideally, an experienced pathologist or a hematopathologist who has been trained adequately in both laboratory hematology and histopathology should do this. He/she should also be competent to assess blood films and BM aspirates and evaluate FCM results. It is preferable that all hematopoietic tissues including peripheral blood, BM aspirate, BM biopsy along with lymph node, spleen, and extranodal lymphoid lesions, are reported by a trained hematopathologist, who shall compile the results of various ancillary techniques. The other option is close cooperation between various disciplines. In any case, a pathologist should not release a trephine biopsy report without ascertaining the opinion on the blood film and BM aspirate (done by him/herself or by another pathologist). If there is any apparent discrepancy between the findings, this should be resolved by a joint examination of the slides of the case or, at the very least, by a telephone conversation between those responsible for reporting the specimens.

Conclusion: Trephine biopsy is an essential technique for the assessment and treatment of patients with a wide range of hematological conditions as well as some other disorders that involve BM. BM trephine is an integral component of diagnosis, staging, and follow-up of HLN. Biopsy is a painful procedure, and due care should be taken by all professionals involved in collection, processing, and reporting of trephine specimens. Trephine should be of adequate volume and integrity, fixed promptly, decalcified fully before being processed, sectioned thinly (2–3 μm), and stained. Routine stains (H&E staining and other special stains) and IHCs must be well standardized with adequate panels. Trephine is used in morphological interpretation and also in the application of various ancillary techniques, including immunohistochemistry, DNA/RNA in situ hybridization, and polymerase chain reaction. It is mandatory to have standard operating procedures for performing a trephine

biopsy, processing of the biopsy specimen, various ancillary techniques, and reporting of the histological sections. In most institutes or hospital settings, BM biopsy and aspirate are mostly reported in different departments, where histopathologists take primary responsibility for bone marrow trephine reporting and hematologists/pathologists report BM aspirates. In few places, both are done by a hematopathologist, which is an ideal situation. There is dire need of qualified hematopathologists who can report benign as well as malignant (bone marrow, nodal, and extranodal) hematopathology. The BM trephine biopsy provides just one piece in this diagnostic jigsaw. Appropriate IHCs are used to detect subtle infiltrates (e.g., intrasinusoidal infiltration as seen in splenic marginal zone B-cell lymphoma and in ALCL). Consideration should be given to take a second opinion from a specialist hematopathologist without wasting time. Who reports the trephine is not important, whether it is a liquid pathologist, surgical pathologist, or a hematopathologist. What is important is to have sufficient expertise available among pathologists reporting trephine specimens in order to ensure that a full range of appropriate investigations is applied in each case, with available detailed clinical and hematological data, and ready access to further specialist advice. Communication and dialog between the hematologist/oncologist and the pathologist is crucial. Ideally, such dialog should occur as the specimen is being reported and generally before multidisciplinary joint clinic (at this time, diagnostic uncertainties should have been resolved). World Health Organization 2008 Classification of HLN [1] requires integration of clinical information, peripheral blood findings, BM aspirate, and trephine morphology, other tissue diagnosis, and data from various ancillary techniques (IHC/FCM, cytogenetic/molecular diagnostics), still a distant reality in India.

Take Home Message: Trephine

1. Aspirate and biopsy are complementary.
2. Good quality fixation, decalcification, and processing are extremely important so as to obtain a good quality H&E section (2–3 μm thick).
3. An accurate diagnosis of HLN requires multidisciplinary approach which includes clinical, biochemical, radiological, hematological parameters with morphological and immunophenotyping analysis.
4. Spend adequate time on scanner/low magnification as it gives vital information about the architecture of BM biopsy, BM cellularity, adequacy of megakaryocytes, any abnormal infiltrates, etc. Cellularity is best commented upon on BM biopsy examination.
5. Higher magnification addresses the cytological features in detail and the presence of parasites, if any.
6. Erythroid cells resemble lymphoid cells, and megaloblasts may resemble blasts. IHC for CD3, CD20, and CD71/CD235 may be performed. LCA (CD45) is strongest in lymphoid cells and may be negative or weakly expressed in erythroid cells. Megaloblasts (as well as mast cells, myeloblasts, plasma cells) might express CD117.
7. Lymphoma and myeloma may present as a nodular deposit. BM aspirate in a few of such cases might reveal a normal marrow differential count. Stain and examine all aspirate smears. Serial deeper cuts on trephine always help.
8. Lymphoid cells constitute approximately 10–20% in a normal healthy adult BM aspirate and biopsy. CD20+ B cells are scanty and singly scattered. CD3+ T cells are also singly scattered and, however, are more in number.
9. Scanty hidden lymphoid infiltrates on trephine may be picked up by CD20 and CD3 stains (for B and T cell, respectively). BM might reveal "missing" B cells (CD20−) in patients of DLBCL (post rituximab). CD19, CD79a, Pax5 are useful B-cell markers in these situations. Overall, CD20 is an extremely important marker. Subtle intrasinusoidal lymphoid infiltrates are well highlighted by IHCs, as the tumor cells in hepatosplenic gamma-delta T-cell lymphoma are picked up by CD3 (double-negative T cells) and tumor cells in splenic marginal zone lymphoma by CD20. These can be eas-

ily missed on H&E images. It is a good idea to do CD3 and CD20 in BM biopsy in cases suspected of hematolymphoid neoplasm.

10. Mimics of lymphomas in BM biopsy are many, from hematogones to benign lymphoid aggregates. Hematogones generally are scattered as single cells in trephine and may express CD10, CD34 and Tdt. It is important to correlate with CBC findings and a meticulous BM aspirate morphology examination followed by flow cytometric immunophnotyping for pattern evaluation. This helps in differentiating hematogones from minimal residual disease in cases of precursor B cell lymphoblastic leukemia (post induction).
11. Plasma cells can mimic morphologically anything under the sun. Minimal or atypical plasma cell infiltrates may be interpreted as either reactive or may be missed. IHC for CD138, cyclinD1, and CD56 may be helpful. Refer text for morphological differences. Plasma cells may be increased in leishmaniasis, aplastic anemia, HL, and angioimmunoblastic T-cell lymphoma apart from plasma cell neoplasm.
12. Bilateral BM biopsies may be performed in staging of neuroblastoma.
13. Aspirate smears may be diluted due to poor technique; hypocellular marrow or else a fibrotic marrow (exclude a possibility of chronic myeloproliferative neoplasm, hairy cell leukemia, CML, HL, and metastatic carcinomas).
14. Granulomas (commonly an infective etiology) may be associated with mast cell lesions or HL.
15. Acute leukemias are best subtyped based on FCI. Blast counts are also best done on aspirate smears. Trephines are best for architecture, cellularity, focal nodular deposits (and tumor deposits), granulomas, fibrosis, interstitial edema, etc.
16. Hypocellular marrow might contain blasts, which can be missed (post treatment, hypocellular AML, and hypocellular MDS). Stains for CD34, glycophorin, and CD117 are helpful.
17. Edematous background may be seen as a treatment-related change.
18. Infidelity of IHC stains is well known. For example, CD56 is expressed by natural killer (NK) cells and their neoplastic counterparts, in AMLs, myeloma plasma cells, small cell carcinomas, and also in cells of other neuroendocrine tumors. CyclinD1 may also be expressed in a plasma cell neoplasm and hairy cell leukemia, in addition to mantle cell lymphoma.
19. Lab must possess adequate IHC panels (including for non-hematolymphoid tumors).
20. Turnaround time should not exceed 3 working days, and 5–7 days where IHC is required. Second opinion may be sought in difficult cases.
21. To err is human, and the pathologist must admit any mistake if it happens.
22. There are gray areas in pathology. Communication between the pathologist and the treating hematologist/oncologist is important.
23. Request for a re-biopsy in case of inadequate/crushed or poorly processed biopsy.
24. A formal/informal training in a busy hematopathology laboratory is highly desirable.
25. Stakeholders for good laboratory practice include physician doing the biopsy (and the anesthetist), OT nurse, medical laboratory technologist and scientists, pathologist, hematologist, administrator, vendor supplying the reagents/equipment to the patient him/herself.
26. Trephine is a painful procedure for the patient and carries some risks; therefore, it should be performed only when there is a clear clinical indication (megaloblastic anemias are best diagnosed based on serum B12/FA levels). Clinical details and the results of relevant laboratory tests including the blood count and blood film features must be known before this procedure is done. It can be performed safely on patients with severe thrombocytopenia, but prolonged pressure may be applied to achieve primary hemostasis.

16.6 Spleen: Interpretation and Lymphomas

Splenomegaly is a common feature in hematolymphoid neoplasms. Mostly spleen is involved by lymphoma elsewhere during its dissemination process. Rarely it may represent the exclusive site of the lymphomatous burden. Thus the designation "primary splenic lymphomas" (PSLs) may be classically restricted to neoplasms fulfilling this latter condition and constitute approximately 6% of all lymphoid neoplasms. However, it must be noted that PSLs commonly involve bone marrow and peripheral blood at presentation.

16.6.1 Normal Spleen

Careful gross evaluation of the specimen and optimal tissue fixation are most important. Because of the high vascularity of the spleen, thin sections are particularly important. Look out for additional lymph nodes with the main splenectomy specimen. The spleen is primarily composed of two distinct regions. The lymphoid tissue of the spleen is called the white pulp (nodules of lymphoid cells) and is associated with the splenic arterial circulation. The central arteries, which arise from trabecular arteries within the fibrous trabeculae, are surrounded by cylindrical cuffs of lymphocytes called periarteriolar lymphoid sheaths (PALS), containing an admixture of B and T cells, with a predominance of CD4+ T cells. Splenic lymphoid follicles (malpighian corpuscles) occur as outgrowths of the periarteriolar lymphoid sheaths. The germinal center is similar to germinal centers seen in lymph nodes. It is surrounded by a mantle zone that is further encased by marginal zone, a cellular layer at the interface between the white and red pulp. The marginal zone is composed of both B and T cells. The red pulp is composed of splenic vascular sinuses and the cords of Billroth, which are made up of splenic macrophages, scattered cord capillaries, venules, and stromal cells. B cells in mantle zone bear surface immunoglobulin, with co-expression of immunoglobulin IgM and IgD. Marginal zone B cells express predominantly IgM, with only a small minority expressing IgD. IgG expression is lacking in these areas and is limited to scattered cells in the red pulp, where rare IgA-containing cells are also found.

16.6.2 Procedure, Collection, and Transport of Specimens: Extranodal Biopsies and Spleen

Measure the weight and describe the gross appearance, including the presence of any focal lesions (e.g., infarcts, nodules, hemorrhage) and gross abnormalities of red or white pulp. The spleen must be sectioned at 3–5 mm intervals, to look for grossly identifiable lesions. Fixation, grossing, and tissue processing are extremely important so as to avoid autolysis of spleen. Splenic FNAC is not used as a diagnostic test. Biopsies from other sites like skin, GIT, etc. are immediately transferred into a suitable fixative (e.g., formalin).

Lymphomas commonly presenting as PSLs are SMZL, splenic lymphomas-unclassifiable (SL-u) [which include splenic diffuse red pulp small B-cell lymphoma (SDRPSBCL) and HCL variant], HCL, LL, B-PLL, T-LGL, and hepatosplenic T-cell lymphoma. Primary splenic presentations of nodal lymphomas are commonly seen in MCL, FL, DLBCL, not otherwise specified, micronodular T-cell/histiocyte-rich large B-cell lymphoma, HL, and PTCL. The commonest PSL is SMZL, and this is a close differential diagnosis of other B-cell lymphomas, namely SL-u, and LPL (waste-basket). All these subtypes belong to low-grade lymphoma category with a wait–and-watch policy for management.

Patterns with involvement of the spleen by lymphoma can be broadly studied in categories of "predominantly red pulp based" and "predominantly white pulp based," both having diffuse and nodular subtypes. Predominantly red pulp involvement is commonly seen with diffuse patterns in HCL, HCL variant, SDRPSBCL, hepatosplenic T-cell lymphoma, acute leukemias,

hemolytic anemias, nonspecific congestion, extramedullary hematopoiesis, etc. It may show a focal or nodular/variable pattern as seen in HL, DLBCL, T-PLL, etc. Similarly, predominantly white pulp involvement may be seen in small B-cell lymphomas (CLL, LPL, SMZL, PTCL, etc.) and may show focal involvement in inflammatory pseudotumor, hamartomas, etc.

Diagnostic Workup in Case of Primary Splenic Lymphomas

1. Complete blood count.
2. Peripheral blood smear and/or bone marrow aspirate examination with FCI.
3. Bone marrow biopsy examination with IHC (CD3, CD4, CD8, CD7, CD5, CD2, CD19, CD20, CD5, CD23, CD10, bcl6, cyclinD1, CD15, CD30, Pax5, EBER, Tdt, CD34) (similar to that for nodal tissues).
4. Cytogenetics and molecular studies (similar to that for nodal tissues).
5. Splenectomy is the last resort (similar IHC panel as above).

Splenic marginal zone lymphoma, the commonest subtype, commonly presents with leukocytosis and splenomegaly. PBS/BM morphology reveals a low-grade B-cell lymphoma that may be labeled as SMZL after excluding other common B-cell lymphomas, namely CLL/SLL, MCL, FL, HCL, LPL (WM), etc. Splenectomy is rarely indicated and reveals a nodular pattern of involvement with a similar IHC profile. SMZL (as per WHO classification) is a B-cell neoplasm comprising small lymphocytes that surround and may replace the splenic white pulp germinal centers, may efface the follicle mantle, and merge with a peripheral (marginal) zone of larger cells, including scattered transformed blasts/immunoblasts; both small and large cells infiltrate the red pulp. Most cases have prominent splenomegaly, and bone marrow and peripheral blood infiltration. Cells in peripheral blood can frequently be recognized morphologically as mature looking lymphoid cells with or without villous projections. SMZL and splenic lymphoma with villous lymphocytes are same entities. SMZL is a diagnosis of exclusion. Tumor cells express pan B-cell markers like CD19 and CD20, however are negative for CD5, CD10, and cyclinD1, and have a low MIB-1 proliferation index. SDRPSBCL reveals a diffuse pattern of involvement with similar morphology of lymphoid cells and IHC profile.

HCL is another common splenic lymphoma, which has a classical morphology on peripheral blood smear, bone marrow aspirate, trephine, and also on splenectomy specimen (widened red lakes). Tumor cells express B-cell markers, show light chain restriction, also express CD11c, CD25, CD103, and CD123, and show BRAFV600E mutation on molecular studies.

16.7 Thymus: Interpretation and Lymphomas

The thymus is located in the anterior mediastinum, where immature T-cell precursors (prothymocytes) that migrate from the bone marrow undergo maturation and selection to become mature, naïve T cells that are capable of responding to antigenic stimuli. It is the site of development of a normal T cell.

Thymus is broadly divided into a cortex and a medulla. The cortex contains cortical epithelial cells and macrophages. Cortical thymocytes (lymphocytes) range in morphology from medium-sized blastic cells with dispersed chromatin and nucleoli located in the outer cortex, to somewhat smaller, more mature-appearing, round lymphocytes located in the inner cortex. Occasional apoptotic bodies and phagocytosis by histiocytes may be seen. The immunophenotype of most cortical thymocytes is that of precursor T cells (TdT+, CD1a+, CD4+, CD8+). The medulla (and perivascular spaces) contains medullary epithelial cells with Hassall corpuscles and dendritic cells. Medullary thymocytes (lymphocytes) are small, morphologically mature-appearing lymphocytes and have immunophenotype of mature T cells (TdT–, CD1a–, CD3+, CD4+, or CD8+). Medulla also contains a particular population of B cells (asteroid cells) with dendritic morphology that expresses mature B-cell markers CD23, CD37, CD72, CD76, immunoglobulin IgM, and

IgD. These cells form rosettes with non-B cells and are cells of origin of primary mediastinal large B-cell lymphoma. Common lymphomas arising in the thymus include T-lymphoblastic lymphoma (T-LL), HL (nodular sclerosis), PMLBCL, and gray zone lymphoma (GZL). Other tumors at this site include thymoma and germ cell tumors. Hodgkin lymphoma has to be differentiated from GZL (intermediate between HL and DLBCL), while T-LL has to be differentiated from normal thymus, thymic cyst, thymoma, etc. Lymphoid cells in normal thymus may contain double-positive T cells, double-negative T cells, and also express Tdt. This may cause an erroneous labeling as blastic lymphoma even in a normal thymic tissue, benign cyst, or thymoma. T-LL presents as a mediastinal mass in a young patient complaining of severe dyspnea as an emergency. On a needle core biopsy, it does not show any neoplastic epithelial component on histopathology and even on IHC will reveal scanty epithelial cells (expressing cytokeratin). T-LL lacks a thick fibrous capsule, lobularity of normal thymus, and also medullary foci. It is seen in young adolescent age group, may show blasts in peripheral blood or bone marrow, and usually shows a uniform immunophenotype among the lymphoid population.

16.8 Extranodal Lymphomas: Interpretation

Although 25–40% of NHL patients present with a primary extranodal lymphoma, in almost every organ in the body, however, common extranodal sites are skin and GIT. Lymphomas arising in extranodal sites vary widely from one extranodal site to another. Some are associated with an underlying immunodeficiency syndrome, autoimmune disease, infection, or other immunologic disorder, or a predilection to affect patients of certain ethnic origins. Common extranodal lymphomas are extranodal marginal zone B-cell lymphoma, mediastinal large B-cell lymphoma, intravascular large B-cell lymphoma, primary effusion lymphoma, plasmablastic lymphoma, DLBCL leg cell type, mantle cell lymphoma, follicular lymphoma, extranodal natural killer/T-cell lymphoma: nasal-type, enteropathy-type intestinal T-cell lymphoma, etc. [24].

16.9 Cutaneous Lymphomas

Most cutaneous lymphomas are of T-cell subtype and show a top heavy infiltrate of atypical lymphoid cells in the upper dermis. Cutaneous T-cell lymphoma (CTCL) is the most common type of cutaneous lymphoma that typically presents with red, scaly patches or thickened plaques that often mimic eczema or chronic dermatitis. CTCL is a group of lymphoproliferative disorders characterized by localization of neoplastic T lymphocytes to the skin. CTCLs are of indolent or aggressive subtypes. The commonest subtype is mycosis fungoides (non-sun-exposed areas).

CTCLs with indolent clinical behavior include the following:

- Mycosis fungoides
- Mycosis fungoide variants and subtypes (e.g., folliculotropic mycosis fungoides, pagetoid reticulosis, granulomatous slack skin)
- Primary cutaneous CD30+ lymphoproliferative disorder (e.g., primary cutaneous ALCL, lymphomatoid papulosis)
- Subcutaneous panniculitis-like T-cell lymphoma (provisional)
- Primary cutaneous CD4+ small/medium-sized pleomorphic T-cell lymphoma (provisional)

CTCLs with aggressive clinical behavior include the following:

- Sézary syndrome
- Adult T-cell leukemia/lymphoma
- Extranodal NK/T-cell lymphoma, nasal type
- Primary cutaneous peripheral T-cell lymphoma, unspecified
- Primary cutaneous aggressive epidermotropic CD8+ T-cell lymphoma (provisional)
- Cutaneous gamma/delta-positive T-cell lymphoma (provisional)

Cutaneous B-cell lymphomas (CBCLs) are a less common version of cutaneous lymphomas, making up about 20–25% of all cutaneous lymphomas. The

most common forms of CBCL are slow growing or indolent variations and respond well to mild treatments.

Newer subtypes have been defined in past few years as cutaneous T-cell lymphomas (two subtypes—CD4 small and medium; CD8 ear type), indolent GI T-cell lymphoma (CD8 equivalent of ear type), mucocutaneous ulcer [25, 26]. Similarly, indolent lymphomas at other sites have been defined as indolent B-cell lymphoproliferations in the blood and bone marrow, indolent B-cell lymphomas (duodenal, pediatric follicular lymphoma, marginal zone that do not progress), early (in situ) lymphomas [2].

16.10 Multidisciplinary Meetings

Data generated from all modes of investigation need to be collated and interpreted in a clinical context. Not every case of lymphoma will have a classical clinical presentation, morphology, immunophenotype, or genetic profile. The reporting pathologist remains responsible for diagnosis and for ensuring appropriate additional investigations are instituted to resolve discrepancies. An individual's experience of all the different investigations, the staining patterns of IPT, the interpretation of FISH, and cytogenetic analysis is useful in weighing up the contribution each investigation makes to the final diagnosis. All diagnoses of hematological malignancy should be discussed in multidisciplinary meetings/boards which include medical, pediatric, and radiation oncologists along with a hematopathologist. Both the diagnosis and clinical management decisions should be recorded in the case file at the meeting. Diagnosis of lymphomas cannot be made without understanding of the clinical background and such multispecialty group meetings help pathologists reach there.

16.11 Reporting

Lymphoma diagnosis on tissue biopsy (nodal, splenic, trephine, as well as extranodal tissue) is done in histopathology department, which may be in a different location from where peripheral blood/BM aspirate is reported (hematology laboratory). It is preferable that both these (tissues and liquid hematopathology) are reported under one roof, so that a final comprehensive report may be generated based on current WHO classification systems. Few clinical hematologists also report BM aspirate smears. The best person to do reporting is a trained and/or experienced pathologist who will report both biopsy and aspirate and should be responsible for integrating the results with ancillary techniques.

The final report should include prognostic and/or predictive markers, if any. These may be assessed by IHC, cytogenetics, or molecular diagnostic methods. For example, CD20 is a predictive marker for B-cell lymphomas. CD38 and Zap70 are prognostic markers in CLL. DLBCL has been further subclassified based on IPT (germinal center or non-germinal center phenotype), based on the site of origin (DLBCL, leg cell type, etc.), based on age (DLBCL of elderly), association with low-grade disease, presence of t(14;18), and expression of BCL2. Though the treatment protocol of all such subtypes of DLBCL is very similar at this moment, it may be a good idea to subtype these for future studies.

The provisional report may be released at first, followed by a final impression along with IHC findings. Supplementary report should follow in case molecular studies/FISH is performed. Kindly note that lymphoblastic lymphoma and Burkitt lymphoma are oncological emergencies, and an early provisional report must be given to the pediatric oncologist. Turnaround time for a lymphoma histopathology report may be 3–4 working days, and 7–8 days when IHC has been performed.

Hematopathologist must be aware of other common causes of abnormal lymphoid proliferations including nonspecific lymphadenitis, EBV infection, tuberculosis, HIV, dermatopathic lymphadenitis, toxoplasmosis, Kikuchi Fujimoto disease, Rosai Dorfman disease, etc.

Pathologists must remember:

1. Common tumors occur at common sites, and the clinical history is extremely important, including site and the age of the patient. Radiological investigations are mandatory as

part of staging and can also give a clue in reaching decisions.
2. Request for a re-biopsy in case of inadequate/crushed or poorly processed tissue.
3. Mimics of lymphomas are many, from benign lesions to other malignant lesions.
4. Second opinion may be sought in difficult cases.
5. To err is human, and the pathologist must admit any mistake if it happens.
6. There are gray areas in pathology. Communication is important between the pathologist and the treating physician/oncologist.

There are many limitations in histopathological diagnosis, including tiny biopsy, crushed sample, necrotic tumor, gray zone lymphomas, etc. There are also problems in reporting core biopsy, like differentiating HL versus mediastinal DLBCL, thymic hyperplasia, and T-LL, BLL, and DLBCL, HL versus DLBCL versus reactive node/viral infections. It may be difficult to opine on small crushed biopsies. Second opinion may be sought, and a re-biopsy should be asked for without wasting time.

Checklist for reporting lymphoma
- Classification according to current WHO classification.
- T- or B-cell phenotype (CD20 and CD3 positive or negative).
- Incorporate IHC in the final report. Mention about the reaction of IHC with the tumor cells (cells of interest) and also the reactive cells in the background. All stains done should be reported.
- Incorporating results of other ancillary techniques.

16.12 Disposal and Storage of Tissues

Each laboratory should follow the local/national laws for waste management/disposal for the remaining specimen, used reagents, garbage, infectious waste, etc. Retention period for tissues may be at least 2 months from the date of dispatch of the final report. These specimens are retrieved, formalin is discarded and the tissue is wrapped in appropriate containers, which may be given to authorized agencies for disposal/incineration. Chemicals like 10% buffered formalin, xylene, and alcohols are hazardous and should be discarded as per guidelines and waste disposal policy of the laboratory.

No diagnostic material should be discarded until all investigations are complete. NABL recommends that paraffin blocks are stored for a minimum of 20 years. Stained slides should be stored for a minimum of 10 years, and preferably longer, especially in case of pediatric patients and in small biopsy specimens where material permitting diagnosis may no longer be contained within the paraffin blocks.

16.13 Fine Needle Aspirate Examination (FNAC)

Cytology is an easy, simple, and economical technique, which is extremely popular among cytopathologists. Experienced cytologists offer an extremely high degree of reliability. FNA material may be used for FCM and molecular tests. Lymphoma diagnosis is best done on a biopsy, and FNAC is not recommended to diagnose lymphomas. In context of lymphomas, FNAC may be useful in the following circumstances:

1. In a known case of lymphoma, for documentation of relapse.
2. In emergency situations, such as in a patient having a mediastinal mass and superior vena cava syndrome, FNAC may be performed before instituting therapy. The sample may be sent for morphology and for FCM. Biopsy should still be advised for proper typing of the lymphoma.
3. FCI is performed for diagnosis and subtyping of HLN; however, subtypes (like HL) may be missed by this technique.
4. FCM may be used to establish a primary diagnosis of hematolymphoid neoplasm when there is no other readily available tissue. In difficult cases, where the biopsy interpretation

is inconclusive, FCM might provide invaluable additional information as elaborate markers are available for FCM. On the contrary, diagnosis and subtyping of myeloid neoplasm and chronic lymphoproliferative disorders (CLPDs) is best done by FCM.

5. FNAC may be performed in suspected cases of tuberculosis.

16.13.1 WHO 2017 Classification of HLN

The WHO 2017 Classification of hematolymphoid neoplasms is expected to be on the stands later this year. Newer defined entities in B-cell lymphomas include small/indolent clonal lymphoid populations, pediatric-type follicular lymphoma, large cell and borderline (gray zone) categories, DLBCL versus BL and "double hit" lymphomas, THRLBCL versus NLPHL, and also a few new genetically defined entities [2]. New entities in T-cell neoplasms include indolent T/NK-cell proliferations, EATL I/II subtypes, which have been further clarified, and few new genetically defined entities. Indolent clonal populations like intrafollicular neoplasia/follicular lymphoma in situ include similar entities like "FL-like B-cells of undetermined significance" and "in situ follicular neoplasia." Other indolent clonal proliferations include mantle cell lymphoma "in situ," indolent NK/T-cell proliferations of the GI tract, CD8+ indolent cutaneous LPD, and seroma-associated ALK-negative ALCL. Pediatric FL are more common than "pediatric-type FL." Pediatric FL are nodal, localized, purely follicular, have high Ki67, are BCL2-negative, and do not reveal any rearrangement of BCL2/BCL6. Not all FL in children are "pediatric type" and such cases may also occur in adults. Follicular lymphoma grade 3B+/− DLBCL with MUM1 expression should be separated from other FL as it behaves more like DLBCL. Similarly, grade 3B FL/DLBCL with IG/IRF4 translocation is a distinct entity within most of DLBCL and few FL, seen in younger patients, at Waldeyer's ring, requires treatment and has a good prognosis.

Diffuse large B-cell lymphoma remains the most intriguing category. In distinguishing GCB from non-GCB types, we may use any of the published IHC algorithms accepted (should be mentioned in the report). Morphologic subtypes may be optional; however, anatomic location is important (CNS, skin, etc.) and should be mentioned in the final report. "Double-hit" B-cell lymphomas are defined as MYC+ with BCL2 and/or BCL6 rearrangements; they have morphology like DLBCL or BL-like (BCL-U). They may be recognized either as a separate category, subdivided by morphology, or as separate categories within DLBCL and BCL-U. IHC for MYC and bcl2 may be performed; however, its co-expression is not clinically actionable at present. We may perform MYC FISH in all new cases of DLBCL, but it is expensive and not available at all places. So FISH may be done in all GC subtype, in double expressor lymphomas (MYC and BCL2 expression) or else high-grade morphology cases of DLBCL. Treatment wise, most of these are still treated with R-CHOP; hence, it is debatable how much is the adequate IHC panel. DHL (2–8% of all DLBCLs) are though treated more aggressively by protocols like dose adjusted R-EPOCH. In the entity EBV+ DLBCL of the elderly, elderly has been removed, and it has been relabeled as EBV + DLBCL NOS. EBV+ mucocutaneous ulcer is a close differential diagnosis (of HL in mucocutaneous sites) and has an indolent behavior (history of immunosuppression). Gray zone between THRLBCL versus NLPHL is still debatable. It is not clear whether diffuse areas/progression in NLPHL may be considered equivalent to THRLBCL. It is important to comment on variant patterns in NLPHL at diagnosis. Few hematolymphoid neoplasms have been defined based on new genetic information like BRAF mutations in HCL, MYD88 mutations in LPL, NOTCH1/2mutations in CLL, MCL, and SMZL, ID3 mutations in BL, BCL-U IG/IRF4 translocations in DLBCL/FL, and DUSP22 translocations/p63 mutations in ALK-negative ALCL.

Next-generation sequencing will be an important tool in the coming years. There are studies to show the contribution of NGS with a consensus gene panel to personalized therapy in DLBCL,

highlighting subtypes' molecular heterogeneity and identifying somatic mutations with therapeutic and prognostic impact [27].

16.14 SOPs and Policies for Tissue Processing

1. Sample accession
2. Grossing procedure: as per standard grossing manuals
3. Fixation
4. Decalcification
5. Tissue processing
6. Embedding
7. Routine staining (hematoxylin and eosin)
8. Mounting procedure
9. Submission of slides
10. Reporting of results (text, comment, impression, signature)
11. Procedure for telephonic reporting
12. Procedure for handling pending reports
13. Slide and block filing
14. Discarding of slides and blocks
15. Medical records
16. Special stains (AFB, GMS, PAS, Congo red, reticulin, Perls', Giemsa, etc.)
17. IHC
18. Karyotyping and FISH studies
19. Molecular studies
20. Internal quality control
21. Proficiency testing program (external quality assurance program)

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