9 Classification, Clinical Manifestations, and Diagnostics of HLH

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Abbreviations

ADV	Adenovirus
CGD	Chronic granulomatous disease
CHS	Chediak-Higashi syndrome
CNS	Central nervous system
CSF	Cerebrospinal fluid
EBV	Epstein-Barr virus
FHL	Familial HLH
HHV	Human herpes virus
HLH	Hemophagocytic lymphohistiocytosis
HPS	Hermansky-Pudlak syndrome
HSCT	Hematopoietic stem cell transplantation
IFN	Interferon
MAS	Macrophage activation syndrome
MRI	Magnetic resonance imaging
NGS	Next-generation sequencing
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PID	Primary immunodeficiency
SAP	Signaling lymphocytic activation molecule-associated protein
sJIA	Systemic juvenile idiopathic arthritis
SLE	Systemic lupus erythematosus
WES	Whole-exome sequencing
WGS	Whole-genome sequencing
XIAP	X-linked inhibitor of apoptosis
XLP	X-linked lymphoproliferative syndrome

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Classification of HLH

Hemophagocytic lymphohistiocytosis (HLH) is a life-threatening hyperinflammatory syndrome. HLH is not a single disease, since a variety of conditions can lead to similar clinical hyperinflammatory phenotypes. The terms used to describe HLH and related syndromes have changed since the original description in 1952 [1]. Ideally, HLH would be classified according to the underlying pathophysiology. However, the pathophysiological basis of HLH varies in different conditions and has not been fully characterized in many disease settings.

Because of its important therapeutic implications, the distinction between "primary HLH" and "secondary HLH" (summarized in Table 9.1) is a clinically relevant issue. This implies the need for a rapid diagnosis of a genetic defect in granule-mediated cytotoxicity. Patients with "primary" HLH require allogeneic

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Table 9.1 Classification of HLH

Primary HLH	**Affected genes**
1. *Familial hemophagocytic lymphohistiocytosis*	*PRF1* (FHL2), *UNC13D* (FHL3), *STX11* (FHL4), and *STXBP2* (FHL5)
2. *Griscelli syndrome type 2*	*RAB27A*
3. *Chediak-Higashi syndrome*	*LYST*
4. *Hermansky-Pudlak syndrome type 2*	*AP3B3A*
5. *X-linked lymphoproliferative disorders*	*SH2D1A* and *BIRC4*
Secondary HLH	**Associated conditions**
1. Infection-associated HLH	**Viral** (including EBV, CMV, ADV, HSV, HHV6, HHV8, VZV, parvovirus B19, influenza, enteroviruses), **bacterial** (including mycobacteria, BCG, *Rickettsia*, *Staphylococcus*, *Klebsiella*, *Ehrlichia*, *Mycoplasma*), **parasitic** (*Leishmania*, *Plasmodium*, and *Toxoplasma*), and rarely **fungal** (*Histoplasma*, *Candida*, and *Cryptococcus*) infections
2. Autoinflammatory and autoimmune diseases	sJIA, NLRC4 mutations, Crohn's disease, SLE, Kawasaki disease (very rarely also familial Mediterranean disease, dermatomyositis, rheumatoid arthritis, sarcoidosis, and systemic sclerosis)
3. Acquired immunodeficiency	Immunosuppressive treatments, HIV infection
4. Malignant diseases	Lymphomas, mostly T/NK cell, but also B-cell lymphomas, and leukemia (rarely solid tumors) cf. Chap. 12 of this book
5. Primary immunodeficiencies (other than primary HLH)	CGD, SCID, CID (WAS, X-MEN syndrome, interleukin-2-inducible T-cell kinase deficiency, CD27 deficiencies (very rarely in X-linked agammaglobulinemia, autoimmune lymphoproliferative syndrome, nuclear factor-kappa B essential modulator deficiency syndrome, CTLA-4 haploinsufficiency, and IFNγ receptor deficiency))
6. Metabolic diseases	Lysinuric protein intolerance, galactosemia, Wolman disease (lysosomal acid lipase deficiency), cobalamin C type methylmalonic aciduria with homocystinuria, propionic aciduria, Gaucher's disease, and hydroxycobalamin deficiency

hematopoietic stem cell transplantation (HSCT) [2–5], and the intensity and duration of immunosuppression needed for disease control are frequently lower in patients with "secondary" HLH.

Primary HLH

The term "primary HLH" is used to denote genetic disorders with a genetic defect in perforin-mediated cytotoxicity. This may be caused by mutations in perforin itself or in genes whose products are involved in the degranulation of perforin-containing granules [6]. In some of these genetic defects, HLH is the key disease manifestation, developing in almost 100% of affected patients – often at birth or in the first few years of life. These diseases are summarized as familial HLH (FHL). Other defects cause syndromic diseases in which HLH is one key manifestation of a more complex syndrome. They are also called immunodeficiencies with albinism. Furthermore, HLH manifesting in the context of immunodeficiency X-linked lymphoproliferative syndrome types 1 and 2 (XLP-1 and XLP-2, with increased vulnerability to Epstein-Barr virus (EBV)) is also classified as "primary." A large proportion of patients with XLP-1 or XLP-2 (55% and 76%, respectively) will experience HLH at some point in life [7]. Infections may trigger the onset of an HLH episode in primary HLH, although in many cases, no infectious agent can be identified. Strong immunosuppression (to achieve remission from hyperinflammatory, active HLH) followed by allogeneic HSCT is clearly indicated in most patients with primary HLH.

Familial Hemophagocytic Lymphohistiocytosis

Familial hemophagocytic lymphohistiocytosis types 2–5 (FHL2–5) constitute a genetically heterogeneous group of rare, autosomal-recessive diseases with an estimated incidence of 0.12 per 100,000 children [8]. Mutations in the *PRF1* (FHL2), *UNC13D* (FHL3), *STX11* (FHL4), or *STXBP2* (FHL5) genes have been found in these patients [9–13]. The underlying genetic defects affect the cytolytic effector protein perforin or other proteins involved in the transport and/or exocytosis of perforin-containing granules to the lytic immunological synapse (as described in detail in Chap. 11 of this book). FHL2 and FHL3 are the most prevalent types, depending on the ethnic origin: 13–50% of patients have FHL2 and 17–41% have FHL3. FHL4 is mostly found in patients of Turkish origin.

Griscelli Syndrome

Griscelli syndrome is characterized by hypopigmentation of the skin and hair, the presence of large clumps of pigment in hair shafts, and an accumulation of mature melanosomes within the melanocytes. Autosomal-recessive defects in the *MYO5A*, *RAB27A*, and *MLPH* genes, respectively, are responsible for Griscelli syndrome types 1, 2, and 3 [14–16]. Only Griscelli syndrome type 2 is associated with an immune disorder that leads to episodes of hemophagocytic syndrome. Rare *RAB27A* mutations have been described that confer a risk for HLH but do not cause albinism.

Chediak-Higashi Syndrome

Chediak-Higashi syndrome (CHS) is caused by autosomal-recessive mutations in the *CHS1* gene (also referred to as lysosomal trafficking regulator, *LYST*) [17]. CHS is also characterized by hypopigmentation of the skin, hair and the eyes. Hair pigmentation abnormalities in CHS differ from the hypopigmentation observed in Griscelli syndromes. Moreover, CHS patients show giant lysosomes in neutrophils and other blood cells, which are diagnostic for this disease.

Hermansky-Pudlak Syndrome

Hermansky-Pudlak syndrome (HPS) is characterized by bleeding problems (due to a platelet function defect) and oculocutaneous albinism. There are ten types of the disorder [18]. HPS2 and HPS10 (caused by mutations in the genes encoding the β3A subunit and the δ subunit of the adaptor protein 3 complex, respectively) are associated with a cytotoxicity defect, but HLH has so far only been observed very rarely in HPS type 2. Its classification as primary HLH is subject to debate. Preemptive HSCT does not appear to be justified in HPS type 2 [19].

X-Linked Lymphoproliferative Disorders

As mentioned above, the primary immunodeficiencies XLP-1 and XLP-2 are associated with a high risk of developing HLH – particularly in the context of an EBV infection. XLP-1 is caused by mutations in the *SH2D1A* gene (also referred to as signaling lymphocytic activation molecule-associated protein, SAP), whereas XLP-2 is caused by mutations in the gene coding for X-linked inhibitor of apoptosis (XIAP) [7]. In both diseases, manifestations other than HLH (such as immunodeficiency, inflammatory bowel disease, or lymphoma) can dominate the clinical picture.

Secondary HLH

The term "secondary HLH" (also referred to as "sporadic" or "acquired HLH") has generally been used to describe patients with (i) a disease fulfilling the clinical diagnostic criteria for HLH and (ii) none of the abovementioned genetic defects. Most patients with secondary HLH suffer from an inherited or acquired underlying disease or are receiving treatment that predisposes them to immune dysregulation, as detailed below. However, the majority of patients with these diseases will never experience HLH. Infections or high inflammatory activity may precede the onset, but not always obvious infectious triggers can be identified.

Infection-Associated HLH

Infections have an important role as triggers in both acquired and inherited forms of hemophagocytic syndromes. Immunocompetent

individuals without any underlying disease may develop infection-triggered secondary HLH. The most common triggers are viral infections and particularly herpes virus infections, especially Epstein-Barr virus. The term viral-associated hemophagocytic syndrome has been used to describe this type of secondary HLH occurring in otherwise healthy individuals. HLH has also been described after infection with numerous different bacteria (including *Brucella*, mycobacterium tuberculosis [20]), fungi, and parasites (especially *Leishmania* [21]). Because of the specific therapeutic consequences, it is of particular importance to recognize that visceral leishmaniosis can present with a clinical picture that is indistinguishable from HLH.

Autoinflammatory and Autoimmune Diseases

Secondary HLH can occur in autoinflammatory syndromes and is most frequently reported in systemic juvenile idiopathic arthritis (sJIA). Many rheumatologists prefer to use the term "macrophage activation syndrome" (MAS), rather than secondary HLH. MAS complicates at least 10% of cases of sJIA, although a much higher proportion of patients (30–40%) show signs of subclinical MAS [22]. MAS can also occur in adult-onset Still's disease. Secondary HLH very rarely occurs in patients with other autoinflammatory syndromes [23, 24]. Recently, a mutation in the nucleotide-binding domain of the inflammasome component NLRC4 was linked to early-onset recurrent MAS [25]. Interestingly, functional assays demonstrated spontaneous inflammasome formation, plus the production of the inflammasome-dependent cytokines IL-1β and IL-18 at levels higher than those seen in cryopyrin-associated periodic fever syndromes (another group of autoinflammatory syndromes).

Furthermore, patients with Crohn's disease are more susceptible to HLH. Since patients with XLP-2 may present with a Crohn's-like disease, the combination of Crohn's disease with HLH should prompt a diagnostic workup for XLP-2 [7], bearing in mind that gastroenterological problems have also been described in patients with *STXBP2* and *NLRC4* mutations.

Patients with autoimmune disorders and those with vasculitis may also suffer from secondary HLH or MAS. In particular, patients with systemic lupus erythematosus (SLE) have an increased risk of developing this complication [26]. Secondary HLH can occur during the acute phase of Kawasaki disease (KD), a hyperinflammatory syndrome associated with vasculitis [27]. When KD patients present with hepatosplenomegaly and an additional laboratory abnormality consistent with HLH (such as cytopenia, liver dysfunction, hyperferritinemia, elevated serum LDH, hypofibrinogenemia, and hypertriglyceridemia), a diagnosis of HLH should be considered. Sporadically, patients with other rheumatologic diseases (such as dermatomyositis, rheumatoid arthritis, sarcoidosis, and systemic sclerosis) develop secondary HLH [28].

Acquired Immunodeficiencies

Secondary HLH can also arise in acquired immunodeficiencies. Treatments with immunosuppressants and certain biologics have been linked to the development of HLH [29]. Patients with sJIA are especially vulnerable when their immunosuppressive treatment is modified. HLH can also arise after chemotherapy and organ or stem cell transplantation. Kidney transplant recipients are at increased risk of HLH (due to immunosuppression), and most such cases are triggered by infection. The mortality rate is over 50% [30]. Screening for a concomitant infection is mandatory, and specific surveillance for EBV and cytomegalovirus infections and for bacterial infections (including mycobacteria) has been recommended in adult patients receiving biologics [31]. Patients infected with HIV alone or in presence of other opportunistic infections or malignancies have an increased risk of developing HLH, and the latter has also been described in a setting of immune reconstitution inflammatory syndrome [32].

Malignant Diseases

Conditions meeting the criteria for HLH may occur in the context of cancer (as discussed in Chap. 12 of this book).

Primary Immunodeficiencies

Secondary HLH may also be a rare but nonetheless clearly associated complication in some genetic diseases. Patients with primary immunodeficiencies (PIDs) other than cytotoxicity defects or X-linked lymphoproliferative disorders may develop an HLH-like disease. PIDs like chronic granulomatous disease (CGD) and combined immunodeficiencies are overrepresented in reports of secondary HLH, relative to other PIDs [33]. CGD is a genetically heterogeneous condition associated with recurrent, life-threatening bacterial and fungal infections. HLH in CGD is mainly associated with bacterial infections. In patients with severe combined immunodeficiency and partial T-cell deficiencies (such as 22q11 microdeletion and Wiskott-Aldrich syndrome), HLH-like episodes tend to occur in the context of a viral infection. Combined immunodeficiencies in which impaired control of EBV infection is a cardinal feature have been associated with EBV-induced HLH in some cases. This includes X-MEN syndrome (X-linked immunodeficiency with magnesium defect, EBV infection, and neoplasia) [34], patients with interleukin-2-inducible T-cell kinase [35], and CD27 deficiencies [36]. Rarely, patients with other PIDs (such as X-linked agammaglobulinemia, autoimmune lymphoproliferative syndrome, nuclear factor-kappa B essential modulator deficiency syndrome, CTLA-4 haploinsufficiency) [37–40] and even IFNγ receptor deficiency [41] develop secondary HLH.

Metabolic Diseases

Patients with metabolic diseases may suffer from secondary HLH. Lysinuric protein intolerance is associated with *SLC7A7* mutations and may be complicated by severe lung disease with pulmonary alveolar proteinosis, renal disease, and an incompletely characterized immune deficiency with HLH [42, 43]. A clinical picture resembling HLH has also been observed in patients with galactosemia, Wolman disease (lysosomal acid lipase deficiency), cobalamin C type methylmalonic aciduria with homocystinuria [44], propionic aciduria [45], Gaucher's disease [46], and hydroxycobalamin deficiency [47].

Clinical Manifestations of HLH

Although the full-blown clinical picture of HLH is quite characteristic, its initial presentation is variable. The most common form is a sepsis-like febrile illness with multiple organ involvement. The cardinal signs of HLH are fever, (hepato) splenomegaly, and pancytopenia. Characteristic laboratory test results include marked hyperferritinemia, hypofibrinogenemia, hypertriglyceridemia, elevated liver enzymes, and hyponatremia. These symptoms can be explained by (i) a high concentration of inflammatory cytokines and (ii) organ infiltration by activated immune cells. In theory, almost every organ can be affected by HLH. With a few exceptions, there are no clinical and laboratory features that allow to differentiate whether the disease occurs in the presence or absence of an underlying genetic defect.

Age at Presentation

Presentation of primary HLH usually occurs in early infancy. Antenatal presentation has been reported and should be considered in the differential diagnosis of nonimmune hydrops fetalis [50–52] and neonatal cytopenia.

Clinical Features

Systemic Symptoms

Most patients have a severely impaired general condition. Prolonged, persistent, non-circadian fever is usually observed in most patients other than neonates and preterm infants (in whom the incidence of fever may be low [48]) and severely ill patients (who may develop hypothermia).

Splenomegaly

Splenomegaly is common in patients with HLH and belongs to the diagnostic criteria. It has been observed in 97% of pediatric and 67% of adult cases [29, 49].

Hepatic Involvement

Hepatic involvement is present in more than half of the patients and may manifest itself as

hepatomegaly, elevated transaminase levels, increased LDH, and/or hepatic cholestasis. Increased triglycerides are also frequently observed and belong to the diagnostic criteria (see below). Occasionally, acute liver failure may even dominate the clinical presentation. In selected patients with secondary HLH-associated liver failure, liver transplantation has been shown to restore good health in an otherwise lethal condition [56]. Fulminant liver failure has also been reported in neonates [57]. Abnormal coagulation is often seen and may be caused by multiple factors, such as liver dysfunction, fibrinogen degradation, low platelet count, and disseminated intravascular coagulopathy. On autopsy, the livers of patients who have died from HLH show periportal lymphocytic infiltration and in some cases evidence of hemophagocytosis [58].

Kidney

Kidney injury may occur in severely ill patients [57, 58]. Glomerulopathy and nephrotic syndrome may develop [30].

Lung

Lung involvement is common in patients with HLH and is suggestive of a poor prognosis [59].

Central Nervous System Involvement

HLH is typically a systemic disease, which can also be associated with variable degrees of CNS involvement (as detailed in Chap. 10). Cases with predominant CNS involvement or initial, isolated CNS involvement are rare [53, 54]. Neurological signs can range from a meningeal irritation to a severe CNS affection, with tetraparesis or epileptic seizures. Microcephaly may develop over time [55]. At the onset of primary HLH, neurological symptoms are mostly associated with abnormal CSF findings and normal brain MRI. Increased CSF cell counts, protein levels, and hemophagocytic features may be observed. MRI abnormalities can be severe but are unspecific. However, it has been shown that relative to patients with acute disseminated encephalomyelitis, patients with HLH are more likely to show symmetric periventricular lesions that do not affect the thalamus or brainstem and do not show T1 hypo-intensity; this may help to distinguish between early lesions in HLH and those observed in other inflammatory diseases of the white matter [55].

Additional Clinical Features

Some patients with HLH-causing gene mutations present with additional clinical features. Patients with Griscelli syndrome type 2, CHS, and HPS typically show pigment abnormalities. In patients with CHS, neurological symptoms (which are more likely to be associated with the causal mutation than with HLH) can occur in early adulthood [60, 61].

Around 30% of patients with XLP-1 develop lymphoma, and XLP-2 patients may suffer from Crohn's-like chronic hemorrhagic colitis and recurrent splenomegaly associated with cytopenia and fever (probably corresponding to minimal forms of HLH) [7]. Both patient groups can develop hypogammaglobulinemia leading to recurrent chest infections.

Laboratory Features

Hematological Signs

Cytopenias are seen in more than 80% patients on presentation [62]. Cytokine-mediated bone marrow suppression might well be more important for the pathogenesis of cytopenia than hemophagocytosis alone. In patients with sJIA and MAS, cytopenias may occur later in the course of the disease because these individuals often have elevated blood counts of neutrophilic granulocytes and thrombocytes prior to developing MAS; in this context, a change over time in these laboratory parameters is more valuable than the absolute values for the early diagnosis of MAS [63].

Ferritin

Very high serum ferritin levels are common in HLH. In the HLH-94 study, the median ferritin level was 2950 ng/mL; ferritin levels greater than 500, 5000, and 10,000 ng/mL were seen in, respectively, 93%, 42%, and 25% of the patients [62]. However, the positive predictive value of

hyperferritinemia as a single parameter for HLH is quite low, in particular in adults [64]. Therefore, one should also consider other possible causes, such as liver disease, hematologic malignancy, or chronic transfusion.

Fibrinogen

Hypofibrinogenemia is most probably caused by increased fibrin degradation by activated macrophages [65, 66]. It may also be worsened in patients with liver dysfunction.

Cytokines

Inflammatory cytokines, such as interferon-gamma (IFN-gamma), tumor necrosis factor/cachectin-alpha, interleukin-6 (IL-6), and IL-1, are augmented in active HLH and contribute to the pathogenesis of the disease [67–69]. Soluble IL-2 receptor (sIL-2R or sCD25) is typically elevated in HLH and serves as a diagnostic marker of the disease [3]. The persistent activation of immune cells that occurs in patients with HLH leads to excessive cytokine production. Recently it has been shown how increased cytokine production is linked to the default in target cell death: prolonged target cell survival is due to failed disengagement of perforin- or granzyme A/B--deficient lymphocytes, increasing mean contact time up to fivefold. The prolonged synapse time leads to repetitive Ca2 + −signaling within the effector cell to cytokine hypersecretion by cytotoxic T cells/NK cells, including IFN-gamma that directly activates macrophages [70].

Serum Sodium Level

Hyponatremia is often present and may be associated with CNS disease. However, pseudohyponatremia (caused by severe hypertriglyceridemia) can also be observed [71].

Diagnosis of HLH

Diagnosing an Episode of HLH

Prompt initiation of treatment is essential for the survival of affected patients. Often the greatest barrier to a successful outcome is late diagnosis. The diagnosis of HLH is difficult because of the rarity of this syndrome, the variable clinical presentation, the similarity to sepsis or flares of an underlying rheumatic disease, and the lack of specific clinical and laboratory findings.

There is no specific test for HLH. Diagnosis is based on the presence of a combination of the various diagnostic features. Hemophagocytosis per se is not sufficient for a diagnosis of hemophagocytic syndrome because macrophages that have engulfed other blood cells (Fig. 9.1) are present in some other conditions and hemophagocytosis can be a late sign even in primary forms of the syndrome. In clinical practice, bone marrow and CSF (or any other biopsy taken in a patient with suspected HLH) should be assessed for hemophagocytosis.

In 1994, the Histiocyte Society proposed a definition of HLH as part of the HLH-94 clinical trial. This definition was later revised for the HLH-2004 trial and currently comprises eight parameters, of which at least five must be met for a diagnosis of HLH [3]. These criteria (with some minor modifications) are listed in Table 9.2.

The diagnostic criteria for HLH as shown in Table 9.2 are appropriate for diagnosing primary HLH and secondary, infection-associated HLH. In some patients with a rheumatic disease, recognition of an HLH episode or MAS may be difficult, due to its resemblance to flares of the

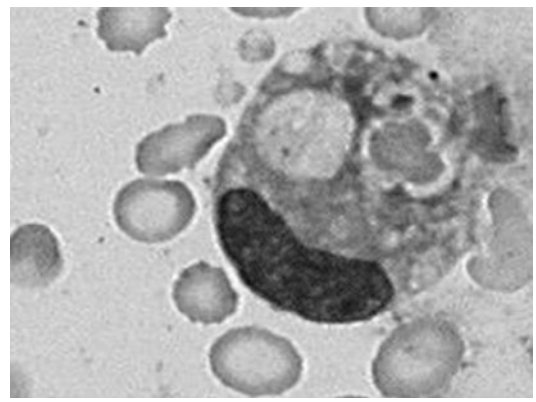

Fig. 9.1 Macrophage with engulfed erythrocytes (i.e., hemophagocytosis) in a bone marrow smear. May-Grunwald-Giemsa stain, light microscope, magnification ×1000 (Adapted from Pachlopnik Schmid and de Saint Basile [92], with permission)

Table 9.2 Diagnostic criteria for HLH

The diagnosis of HLH can be established if **(A)** and **(B)** are fulfilled
A. A molecular diagnosis consistent with HLH: disease-causing mutations in *PRF1*, *UNC13D*, *Munc18-2*, *STX11*, *RAB27A, LYST, SH2D1A*, or *BIRC4*
B. Five out of the eight criteria listed below are fulfilled:
1. Fever ≥38.5
2. Splenomegaly (palpable below costal margin or increased size by imaging)
3. Cytopenia (affecting ≥2 out of the 3 lineages):
Hemoglobin (<90 g/l; in newborns, <100 g/l) Neutrophilic granulocytes ($<1.0 \times 10^9$/l) Platelet count ($<100 \times 10^9$/l)
4. Hemophagocytosis (in the bone marrow or CSF)
5. Hyperferritinemia (≥500 μg/l)
6. Hypertriglyceridemia (fasting level, ≥3.0 mmol/l) or hypofibrinogenemia (≤1.5 g/l)
7. Elevated soluble CD25 (≥2400 U/ml)
8. Decreased NK-cell cytotoxicity

Adapted from Henter et al. [3]

underlying rheumatic disease. The fibrinogen level and the absolute neutrophil and thrombocyte counts may be misleading in patients with sJIA who experience MAS. In sJIA (in the absence of MAS), elevation of these parameters is typical [72]. In contrast, MAS leads to a relative decrease in fibrinogen and cell counts and might therefore be underdiagnosed since it does not lead to hypofibrinogenemia, neutropenia, or thrombocytopenia in absolute terms. It is noteworthy that sJIA features spikes of fever, whereas MAS tends to be associated with a persistently elevated body temperature.

To aid with the diagnostic process, consensus criteria for the classification of MAS in patients with sJIA have been published [73] as detailed in Chap. 13. The physician must check that the laboratory parameters cannot be explained by other aspects of the patient's condition, such as concomitant immune-related thrombocytopenia, infectious hepatitis, visceral leishmaniasis, or familial hyperlipidemia. Patients with sJIA and recurrent MAS should be screened for primary HLH; they may profit from targeted treatment with IL-1 antagonists or tocilizumab. Autologous hematopoietic stem cell transplantation following intensive immunosuppressant therapy has been performed in some patients with severe treatment-resistant sJIA. This may include individuals with severe, recurrent MAS [74]. However, HSCT currently remains an experimental therapy targeting the underlying disease in very selected cases rather than an accepted treatment modality for MAS itself.

The assessment of a bone marrow aspirate will help to rule out hematological neoplasia. *Leishmania* infection should be searched by PCR in a bone marrow specimen, since false-negative results may result from PCR of peripheral blood, serology, bone marrow microscopy, and bone marrow culture [21]. In primary HLH, a marrow with normal or increased cellularity (especially for the erythropoietic lineage) is typical. In contrast, MAS in sJIA tends to be associated with an increase in the granulocytic lineage. However, hemophagocytosis in bone marrow may not be present, especially at the beginning of an HLH episode.

Material obtained from other organs (such as CSF) may also be of value in the diagnostic procedure. However, hemophagocytosis is not an obligatory diagnostic criterion, and we do not recommend the performance of liver biopsies in patients with active HLH (due to potential bleeding).

The CSF should be analyzed for of cell count, protein level, and hemophagocytic features on a cytospin preparation. Changes in CSF may precede changes in brain imaging.

Diagnosing the Underlying Disease Associated with an HLH Episode

In recent years, the introduction of new immunologic and genetic analyses has made it easier and faster for the physician to diagnose patients with suspected primary HLH [75]. For example, the identification of novel HLH-associated genes has increased the possibilities to establish a definite diagnosis of primary HLH [11–13]. The results of more specific immunological tests (which may be available within 1–3 days) allow the rapid identification of patients in need of HSCT and

can guide the priorities in targeted genetic analysis [75]. In addition, next-generation sequencing allows more comprehensive genetic analyses and helps to identify novel genes that predispose to HLH [76]. Hence, diagnostic approaches are changing, and the most efficient algorithm has yet to be determined.

Once an HLH episode has been diagnosed, immunologic screening is valuable for guiding the subsequent workup. A possible diagnostic flow sheet is shown in Fig. 9.2. Deficient protein expression or NK-cell degranulation should prompt targeted genetic testing. In some cases, the clinical context can provide additional clues for the diagnosis of inherited vs. acquired disease. Inherited hemophagocytic syndromes are more frequent among young children (especially <1 year of age) and in cases of severe and/or recurrent disease, parental consanguinity, a family history suggestive of an X-linked disease, and pigmentary anomalies. However, it must be noted that (i) acquired forms can also start early in life and can also be severe and can be recurrent in sJIA and other autoinflammatory diseases, and (ii) inherited forms can start later in life and may be sporadic, attenuated in severity, or even oligosymptomatic (e.g., with neurological involvement alone). Therefore, even milder forms of HLH, HLH in adult male patients (especially those with a family history suggestive of an

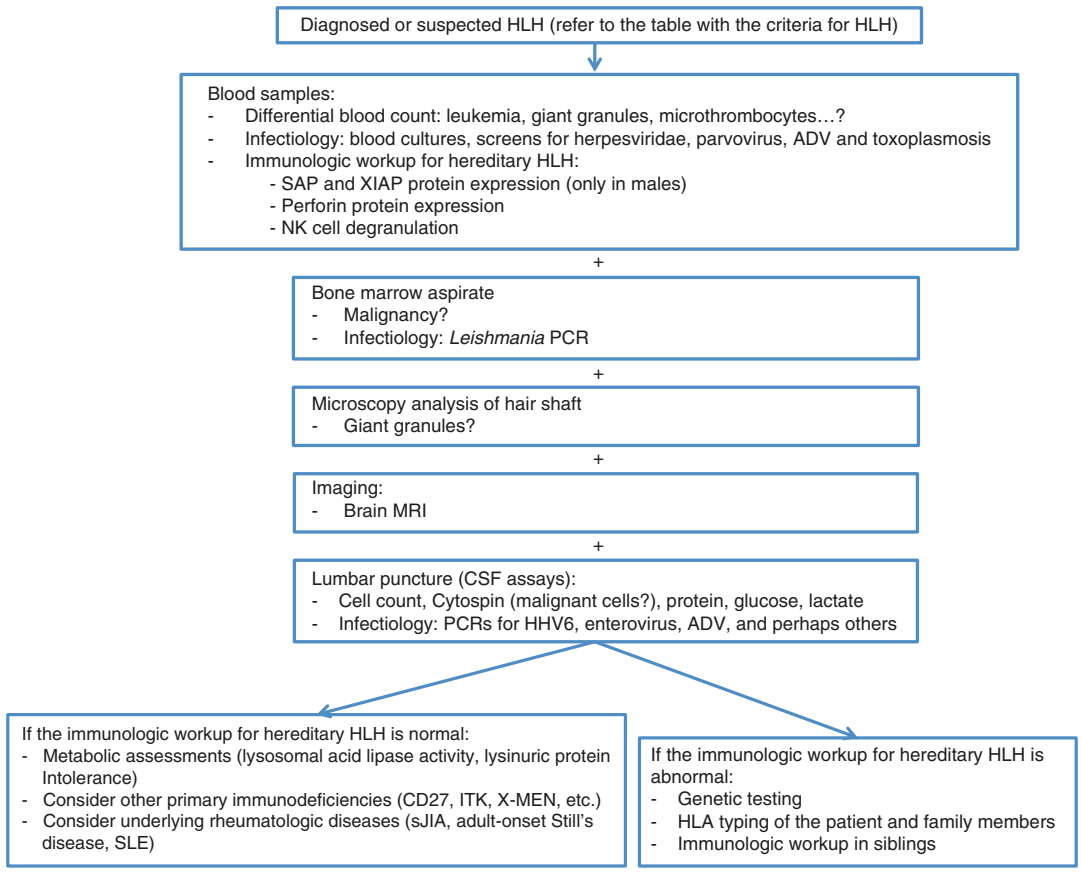

Fig. 9.2 Immunologic testing may support a diagnosis of primary HLH and provide functional data, whereas gene sequencing (typically requiring 3–8 weeks) may define the specific mutations. If a repeatedly abnormal test suggests an underlying functional abnormality, genetic testing should include sequencing of introns, should consider deletions, and should encompass sequencing of all relevant genes (including the genes associated with albinism even in the absence of this symptom). Normal immunological test results cannot fully exclude a genetic disease, although this is very rare in experienced labs

X-linked disease), and neurological signs with reasonable grounds for suspecting HLH are valid indications to perform diagnostic tests for defects in cell cytotoxicity by degranulation and protein expression assays. However, in these cases the possible benefits of the diagnostic tests should also be weighed against the costs of unnecessary tests and possibly resulting unnecessary follow-up and even treatment.

In view of the iatrogenic lymphopenia that can be induced by the subsequent treatment, immunological tests should be performed early in the disease course. However, valid results can be obtained even under HLH-2004 therapy. Microscopic hair analysis (Fig. 9.3) is a simple analysis and should be carried out in patients with a silvery shine of the hair (even in those with dark hair) or patients with fair hair (because the silvery shine can be very difficult to recognize in these cases). Furthermore, giant granules should be searched for in the differential blood count because they are pathognomonic for CHS.

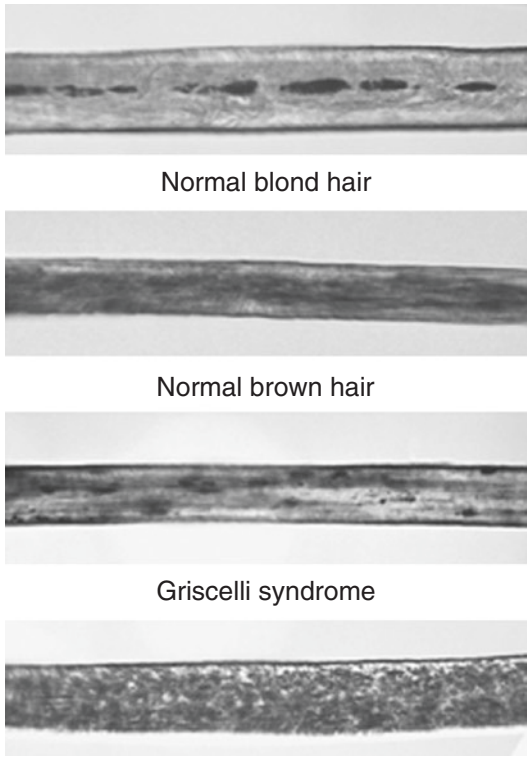

Fig. 9.3 Normal hair shafts (*upper panels*) and characteristic large clumps of pigment in the hair shaft of patients with Griscelli syndrome and Chediak-Higashi syndrome (*lower panels*). Light microscope, magnification × 250 (Adapted from Pachlopnik Schmid and de Saint Basile [92], with permission)

Sequential immunological testing, followed by a genetic diagnostic approach, provides the basis for a rapid transplant decision and timely preparation for allogeneic HSCT. Targeted sequencing is currently the most widely used approach. Immunological screening can identify patients with primary HLH but requires specific expertise. With advances in next-generation sequencing, genetic approaches may prove to be technically easier and may prevail in the future.

Patients should also be screened for autoimmune diseases or malignancy with a detailed clinical history, a physical examination, and other appropriate analyses.

In a patient with a PID, an episode of secondary HLH may occur when the PID is diagnosed or may even reveal the PID. PIDs other than disorders of cytotoxicity or XLP are therefore a relevant differential diagnosis in patients presenting with HLH syndrome [33]. It has been reported that patients with an underlying T-cell PID have a significantly higher ferritin/sCD25 ratio (>10, on average) than patients with FHL and CGD patients (<1, on average).

In a patient with HLH, it is essential to understand the underlying etiology and to screen extensively for infectious triggers. Extensive immunosuppressive treatment may be needed to control the immune dysregulation. Treatment with immunosuppressants in the absence of anti-infective medications may have serious consequences ranging from unnecessary overtreatment [21] to a potentially lethal outcome.

Screening for infectious agents such as EBV, cytomegalovirus, herpes simplex virus, adenovirus, parvovirus B19, mycobacteria, and *Leishmania* is recommended, since most of these agents are amenable to treatment. The detection of EBV infection has important therapeutic implications because elimination of the main EBV reservoir by B-cell-directed treatment is an important component of therapy in all patients with EBV-associated HLH [77].

Degranulation Test

Testing the patient's cells for their degranulation capability is becoming a routine procedure in suspected HLH. The results can be obtained within less than 2 days. In immunocompetent individuals, when cytolytic cells such as cytotoxic T lymphocytes (CTL) and natural killer (NK) cells recognize a target cell, they kill the target cells by secreting perforin and granzymes [78–81]. Mutations in the genes *UNC13D, STX11, STXBP2*, *RAB27A, LYST*, and *AP3B1* all affect either vesicle loading, vesicle maturation, or vesicle fusion with the plasma membrane [13, 16, 82–86], thereby preventing proper target cell killing by cytolytic cells. The degranulation test thus determines whether the secretory pathway for lytic proteins is functional.

In the degranulation test, peripheral blood mononuclear cells (PBMCs) are activated by incubation with K562 cells. As K562 cells do not express MHC class I molecules, they are recognized as "missing self" and subsequently killed by NK cells by secretion of their cytotoxic molecules. Successful degranulation of NK cells can be determined based on the presence of CD107a (LAMP1) on the cell surface [87]. The protein CD107a is normally only found on lysosomal membranes but can be detected on the surface of cytotoxic cells after fusion of cytotoxic vesicles with the plasma membrane. The degranulation test is thus based on the appearance of the lysosomal membrane protein CD107a on the surface of activated NK cells, measured by flow cytometry (Fig. 9.4).

Studying the expression of CD107a on NK cells of patients suffering from different forms of primary HLH and healthy individuals allowed Bryceson et al. to evaluate normal and pathogenic degranulation percentages [75]. According to their work, no degranulation defect is present with values above 10%. Degranulation values between 5% and 10% are not conclusive and a repetition of the assay is recommended. Degranulation values below 5% are, however, very suggestive of an inherited defect and warrant further genetic investigation of the known mutations affecting degranulation.

It is important to be aware that functional degranulation still occurs when the genetic cause of HLH can be attributed to either *PRF1*, *SH2D1A*, or *XIAP* [75]. The degranulation assay should therefore routinely be accompanied by an intracellular staining of CTLs or NK cells for perforin, SAP, and XIAP – which can all be performed on the same blood sample used for the degranulation test. In combination, the degranulation test and intracellular staining can detect all currently known HLH-causing gene mutations.

Genetic Analysis

Fulfillment of at least five defined HLH (HLH-2004) criteria out of a set of eight is sufficient for clinical HLH diagnosis. Immunological and genetic tests are needed for diagnosis of inherited HLH. Nowadays Sanger sequencing of known mutations is a relatively cheap, robust, and

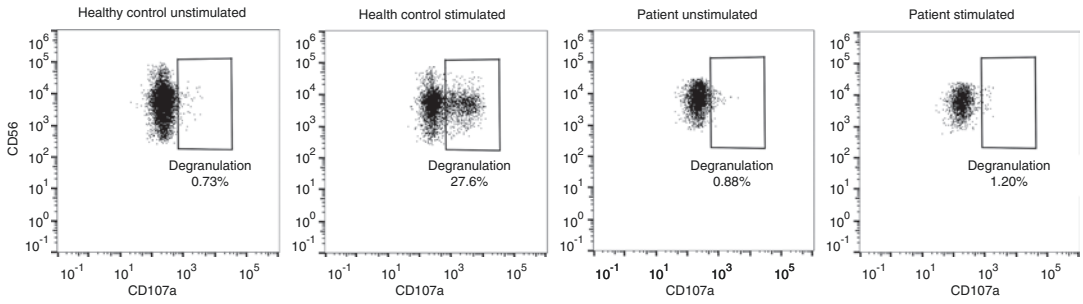

Fig. 9.4 Degranulation test of healthy control and affected patient. Dot plots of NK cells (CD3- CD56+) showing degranulation (CD107a+) after stimulation with K562 cells in the healthy control (*left panels*). In the affected patient with Munc13-4 deficiency, degranulation is below 5% (*right panels*)

accepted method for detecting genetic mutations. Minute amounts of DNA extracted from blood are sufficient for sequencing. A drawback of Sanger sequencing is that being a targeted sequencing method, only known mutations are detected. The detection of novel disease-causing mutations is possible by sequencing the whole gene instead of only the regions with known mutations, but this may exaggerate the cost and effort. The advent of next-generation sequencing and its increasing affordability may however fully replace Sanger sequencing in the near future [88, 89].

In contrast to Sanger sequencing, next-generation sequencing (NGS) methods allow to cover large parts of the genome [90]. Whole-exome sequencing (WES) and whole-genome sequencing (WGS) are the best-known methods, and with targeted NGS, sequencing, e.g., all genes linked to immunological disorders, is possible. Compared to Sanger sequencing, NGS currently depends on high throughput for cost-efficiency, and the duration from taking the blood sample to obtaining the sequencing results is longer. Therefore today, WES is especially used for the discovery of novel disease-causing mutations, as it detects mutations in all protein coding regions of the genome [18, 91]. However, filtering all the detected variants and assigning a specific mutation to a patient's phenotype is very challenging and time-consuming. Nevertheless, as NGS is further improved, WES or a customized NGS gene panel might become an important screening tool for HLH patients or other patients with a suspected immunodeficiency [88].

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