Genetics of Familial Hemophagocytic Lymphohistiocytosis (HLH)

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KEYWORDS

• HLH • Familial • Genetics • Variant • Diagnosis • Genetic testing
• Genotype-phenotype correlations • Genetic counseling

KEY POINTS

- The familial forms of hemophagocytic lymphohistiocytosis (fHLH) are associated with germline defects in genes required for lymphocyte cytotoxic activity and immune regulation.
- When evaluating for fHLH, genetic counseling and timely comprehensive germline genetic/genomic testing are imperative.
- Genetic test results should be interpreted in the context of each patient's specific clinical scenario, including laboratory results, especially those from immunologic functional studies.
- Continuing advances in understanding the genetic causes of fHLH would enable further optimization in diagnostic, prognostic, and therapeutic approaches.

INTRODUCTION

Hemophagocytic lymphohistiocytosis (HLH) is a rare, life-threatening immunological syndrome characterized by the uncontrolled activation of CD8 T lymphocytes, natural killer cells, and macrophages, which secrete excessive amounts of proinflammatory cytokines. HLH can be inherited (also known as primary or familial HLH [fHLH]) or it can be acquired (secondary [sHLH]). The familial forms of HLH were originally mapped

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Abbreviations	
CHS	Chédiak–Higashi syndrome
EBV	Epstein-Barr virus
fHLH	Familial hemophagocytic lymphohistiocytosis
GS2	Griscelli Syndrome type 2
HLH	Hemophagocytic lymphohistiocytosis
HPS2	Heřmanský -Pudlák syndrome
HSCT	Hematopoietic stem cell transplant
LYST	Lysosomal trafficking regulator
NGS	Next-generation sequencing
NK	Natural killer
PIDs	Primary immunodeficiency disorders
RICD	Restimulation-induced cell death
SAP	SLAM-associated Protein
sHLH	Secondary hemophagocytic lymphohistiocytosis
SLAM	Signaling lymphocytic activation molecule
VUS	Variants of uncertain significance
WES	Whole-exome sequencing
WGS	Whole-genome sequencing

to 5 distinct genetic loci, with the causal genes identified for 4 of these conditions, including *PRF1* (FHL2), *UNC13D* (FHL3), *STX11* (FHL4), and *STXBP2* (FHL5). Identification of the fHLH genes has greatly enhanced our understanding of disease pathogenesis which is now known to result from impaired CD8 T cell and natural killer (NK) cell cytotoxicity. The inability of CD8 T or NK cells to effectively eliminate target cells, including infected, malignant, or activated antigen presenting cells, leads to a build-up of these cells, as well as the continued proliferation of T cells and macrophages, which release proinflammatory cytokines and cause widespread tissue damage and death if not properly recognized and promptly treated.

The diagnosis of fHLH can be challenging due to the similarities it shares with other inflammatory, infectious, and malignant conditions. Nevertheless, it is imperative to determine whether an individual with concerning signs and symptoms has fHLH versus sHLH because fHLH can only be cured via allogeneic hematopoietic stem cell transplant (HSCT). It is also important to distinguish between fHLH and various fHLH disease mimics, which include inborn errors of the immune system,[1] such as lymphoproliferative disorders (*SH2D1A, XIAP*), complex immune deficiencies (*RAG1, IL27RA*), hereditary defects affecting inflammasomes (*NLRC4, NLRP3*), and metabolic dysfunction (*SLC7A7, GBA, LIPA*). Fortunately, advances in and utilization of genomic technologies, including next-generation sequencing (NGS) and microarray analysis, have made it possible to distinguish between these disorders. In recognition of the importance of establishing a genetic diagnosis, finding mutations in the fHLH genes is included as one of the diagnostic criteria for the disease.[2] Thus, every effort should be made to reach a prompt and accurate genetic diagnosis, essential for managing and treating fHLH, as it allows for appropriate early and potentially curative therapies, and consideration of genetic counseling, testing, risk prediction, and family planning options for affected families.

THE GENETICS OF FAMILIAL HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS

The genetic background of classical fHLH is a defect in one of the genes responsible for the cytotoxic granule pathway (**Fig. 1**). These granules contain perforin and granzymes. Upon release, perforin permeabilizes the cell membrane of the target cell,

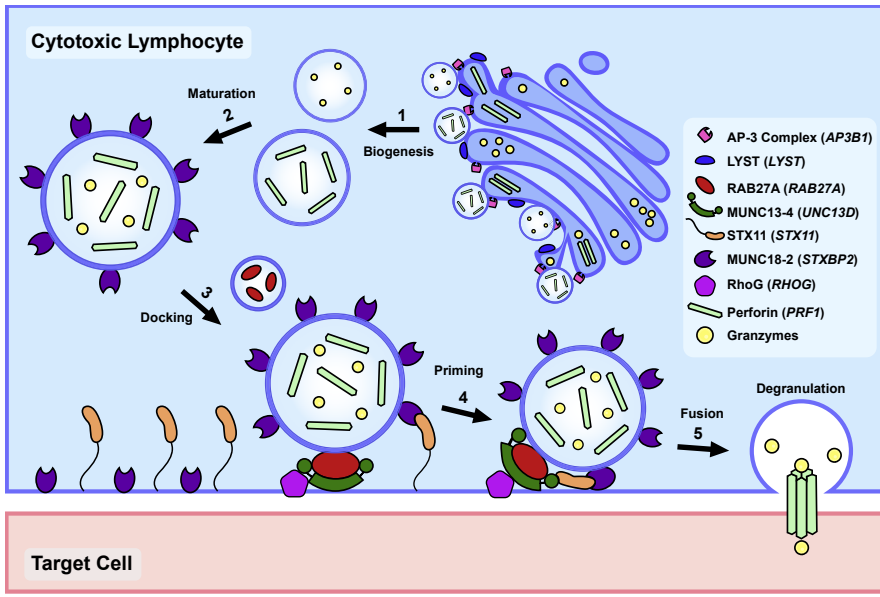

Fig. 1. Cytotoxic lymphocyte degranulation pathway. Upon target cell engagement, the degranulation pathway in cytotoxic lymphocytes undergoes a series of coordinated steps. (1–2) Cytotoxic granule biogenesis and maturation: LYST protein and the AP-3 protein complex function at the Golgi membrane to facilitate the production of cytotoxic granules that contain the correct complement of cytotoxic proteins as well as the lysosomal proteins required for appropriate intracellular localization of the granules. (3–4) Docking and priming: Following transportation of cytotoxic granules to the immunologic synapse, they require tethering to the plasma membrane, which is mediated by the coordinated activity of a number of proteins, including RAB27A, STX11, and MUNC18-2. Once properly localized to the immunologic synapse, MUNC13-4 and RhoG facilitate priming, a process that makes cytotoxic granules competent for exocytosis. (5) Fusion and pore formation: Properly docked and primed cytotoxic granules fuse with the plasma membrane at the immunologic synapse, facilitating translocation of their internal contents across the immunologic synapse and toward the target cell. Perforin then forms a protein pore in the target cell membrane, through which granzymes enter to induce apoptosis.

enabling granzymes to enter and trigger apoptotic death, while the cytotoxic effector cell's membrane is not affected.[3] In fHLH, cytotoxic T and NK-cells cannot kill target cells, yet their ability to proliferate and produce cytokines remains intact. Released cytokines stimulate macrophages and other immune cells, resulting in positive feedback loops leading to cytokine storm, and finally, full-blown HLH. Pathologic mutations in genes responsible for the formation, trafficking, and release of cytotoxic granules are typically germline and recessive. A dominant-negative effect from a mutated protein is possible but rarely observed.[4] Therefore, fHLH is caused by mutations in perforin and specific genes acting upstream (**Table 1**), but establishing a link between the given mutation and patient phenotype may be challenging.

PRF1 (FHL2)

Encoded by the gene *PRF1*, perforin is predominately expressed by lymphocytes of the CD8 T-cell and NK-cell lineages. Perforin is stored in specialized secretory lysosomal vesicles commonly termed cytotoxic granules. Upon recognition of a susceptible target cell, perforin can form a pore that perforates the target cell membrane which

Table 1
Genes associated with familail HLH

Locus-Based Name	Disease	Related Gene	Gene Function	References
FHL2	Perforin deficiency	*PRF1*	Pore forming protein	Stepp et al,[48] 1999; Lopez et al,[49] 2013
FHL3	Munc13-4 deficiency	*UNC13D*	Vesicle priming and secretion of lysosome	Boswell et al,[50] 2012
FHL4	Syntaxin-11 deficiency	*STX11*	Vesicle intracellular trafficking/ membrane fusion	zur Stadt et al,[51] 2005; Bryceson et al,[11] 2007
FHL5	Munc18-2 deficiency	*STXBP2*	Vesicle intracellular trafficking/ membrane fusion	Spessott et al,[4] 2015

serves as a conduit for the entry of granzymes into the target cell (see Fig. 1).[5] Perforin is essential for cell-mediated cytotoxicity. A lack of perforin protein abrogates cytotoxic activity at a very early age. Besides perforin, cytotoxic granules contain granzymes and other proteins that promote target cell death. Surprisingly, although granzymes are the main mediators of target cell death in this pathway, mutations in the genes encoding granzymes are not among those responsible for fHLH. This is likely because the granzymes are the products of 5 separate genes (A, B, H, M, and K), which can compensate for one another in terms of function.[6]

UNC13D (FHL3)

The Unc-13 Homolog D (*UNC13D*) gene encodes the protein MUNC13-4 (mammalian homolog of *C. elegans* uncoordinated gene-13). Through its interaction with other proteins, MUNC13-4 functions to tether and dock cytotoxic granules at the plasma membrane. The related genes *UNC13 A*, *B*, and *C* have roles in the neurological synapse, while *UNC13D* is primarily involved in the immune response. Nevertheless, patients with MUNC13-4 deficient FHL3 have more frequent central nervous system involvement than those with FHL2.[7] And heterozygous mutations in *UNC13D* have been found in patients with systemic juvenile idiopathic arthritis and autoimmune lymphoproliferative syndrome.[8]

STX11 (FHL4)

The *STX11* gene encodes syntaxin 11—a membrane-anchored protein involved in the docking and release of cytotoxic granules. NK cells from FHL4 patients fail to degranulate when encountering susceptible target cells. Patients with germline mutations in S*TX11* have a later onset and milder clinical course than patients with germline mutations in *PRF1*.[9,10] Interestingly, treatment with interleukin 2 (IL-2) partially restores degranulation and cytotoxicity in FHL4 patients' cells. This effect is also observed, but to a lesser extent, in FHL3 but not in FHL2 cells, as IL-2 facilitates degranulation but cannot reverse perforin defects.[11]

STXBP2 (FHL5)

Syntaxin binding protein 2 (known as MUNC18–2) binds to Syntaxin 11 during docking and exocytosis of cytolytic granules. It acts as its chaperone, and loss of MUNC18-2 impairs the expression and/or localization of Syntaxin 11 in the plasma membrane.[12] Curiously, MUNC18-2 localization does not change in FHL4 cells that lack syntaxin

11.[12] The mechanism of FHL5 is more complex than the indirect loss of Syntaxin 11 because MUNC 18-2 also has a separate role in degranulation, as it is required for complete membrane merging during exocytosis.[13] Restoration of cytotoxic function by IL-2 is possible in FHL5, but mutations resistant to this effect have also been described.[14] The age of presentation may vary even within the same family, and clinical manifestations may include colitis, bleeding disorders, and hypogammaglobulinemia.[15] Bleeding may be associated with impaired platelet activity because this protein is also required for platelet secretion.[16]

GENETIC DEFECTS UNDERLYING FAMILIAL HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS MIMICS

The fHLH mimics are disorders characterized by HLH that are caused by genetic defects other than those discussed above. Among these are included the conditions below and in **Table 2**, although many other disorders exist with similar propensity to developing hyperinflammation.

OTHER GENES IN THE GRANULE CYTOTOXICITY PATHWAY

Some genes involved in the cytotoxic granule pathway have more pleiotropic functions, and their mutations cause distinct constitutional syndromes with a high risk of developing HLH at a young age. For example, mutations *RAB27A*, *LYST*, and *AP3B1* share a common feature of albinism due to defects affecting melanocyte function.

RAB27A

RAB27A is a member of the Rab GTPase family, expressed in many tissues, and regulates vesicular transport and organelle dynamics. The RAB27A protein is involved both in melanosome transport in melanocytes (binding with myosin for transport along microtubules) and in tethering and docking of cytotoxic granules to the membrane (together with MUNC13-4). Mutations in the gene encoding this protein cause Griscelli Syndrome type 2 (GS2) with partial albinism (cases of sine albinism were also described),[17] immunodeficiency, and high HLH risk—including early or isolated CNS involvement.[18]

LYST

Lysosomal trafficking regulator (*LYST*) gene is an important molecule for the regulation of membrane dynamics and intracellular trafficking of lysosomes and lysosome-related organelles. Defects in *LYST* cause Chédiak–Higashi syndrome (CHS), characterized by oculocutaneous albinism, immune deficiency, coagulation defects, and progressive neuropathy. Over 80% of patients with CHS reach the hyper-inflammatory *accelerated phase* of the disease, which clinically mimics fHLH. Currently, there is no clear understanding of the cellular and molecular mechanisms underlying *LYST* function; however, studies have demonstrated that *LYST* plays important roles in microtubule dynamics, granule fusion and fission events, and membrane docking facilitated by SNARE complex proteins. Abnormalities in these functions could affect endolysosomal trafficking and the immune response, resulting in the development of clinical HLH.[19,20]

AP3B1

Mutations in the *AP3B1* (Adaptor related Protein complex 3 Beta 1 subunit) are responsible for the second type of the Heřmanský -Pudlák syndrome (HPS2), which

Table 2
Genetic conditions mimic familial HLH

Conditions	Gene	Disorder	OMIM	MOI	References
Granule-mediated cytolytic pathway-with/without pigmentary disorders	*LYST*	Chediak-Higashi syndrome	606897	AR	Barbosa et al,[20] 1996
	RAB27A	Griscelli syndrome type 2	607624	AR	Ménasché et al,[52] 2000
	AP3B1	Hermansky-Pudlak syndrome 2	603401	AR	Jessen et al,[53] 2013; Wenham et al,[54] 2010
	RHOG	RHOG-associated hyperinflammatory disorder 8	179505	AR	Kalinichenko et al,[22] 2021
Lymphoproliferative disorders	*CD27*	Lymphoproliferative syndrome 2	186711	AR	Salzer et al,[55] 2013
	CDC42	Neonatal onset of pancytopenia, autoinflammation, rash, & episodes of HLH (NOCARH)	616737	AD	Lam et al,[56] 2019
	ITK	Lymphoproliferative syndrome 1	613011	AR	Huck et al,[57] 2009
	MAGT1	Immunodeficiency, XL, w/ magnesium defect, EBV infection, & neoplasia	300853	XL	Li et al,[58] 2014
	SH2D1A	X-linked lymphoproliferative disease 1	308240	XL	Booth et al,[59] 2011
	XIAP	X-linked lymphoproliferative disease 2	300635	XL	Rigaud et al,[25] 2006
Inflammasome activation disorders	*NCKAP1L*	NCKAP1L-associated hyperinflammatory disorder 5	618982	AR	Castro et al,[60] 2020
	NLRC4	Autoinflammation w/infantile enterocolitis	616050	AD	Canna et al,[41] 2014
	NLRP3	Familial autoinflammatory syndrome	606416	AD	Alehashemi et al,[61] 2020
	RC3H1	RC3H1-associated hyperinflammatory disorder 7	618998	AR	Tavernier et al,[62] 2019

Inborn errors of metabolism	*BTD*	Biotinidase deficiency	253260	AR	Kardas et al,[63] 2012
	COG6	COG6-CDG & HLH	614576	AR	Althonaian et al,[64] 2018
	CTSA	Galactosialidosis	256540	AR	Olcay et al,[65] 1998
	G6PC1	G6PC1-related glycogen storage disease type I	232200	AR	Düzenli Kar et al,[66] 2019
	GALT	Classic galactosemia	606999	AR	Kundak et al,[67] 2012
	GBA1 (GBA)	Gaucher disease	606463	AR	Sharpe et al,[68] 2009; Anderson & Taylor,[69] 2020
	HADHA	LCHAD deficiency (See Long-Chain Hydroxyacyl-CoA Dehydrogenase Deficiency/ Trifunctional Protein Deficiency.)	609016	AR	Erdol et al,[70] 2016
	LIPA	Wolman disease (See Lysosomal Acid Lipase Deficiency.)	613497	AR	Taurisano et al,[71] 2014
	MMUT	MMUT-related isolated methylmalonic acidemia	609058	AR	Gokce et al,[72] 2012
	MMACHC	Cobalamin C disease (See Disorders of Intracellular Cobalamin Metabolism.)	277400	AR	Wu et al,[73] 2005
	mtDNA deletion	Pearson syndrome (See Mitochondrial DNA Deletion Syndromes.)	557000	Mat	Wild et al,[74] 2020
	NPC1	NPC1-related Niemann-Pick disease type C	257220	AR	Karaman et al,[75] 2010
	PCCA	PCCA-related propionic acidemia	606054	AR	Gokce et al,[72] 2012
	SLC7A7	Lysinuric protein intolerance	603593	AR	Mauhin et al,[76] 2017
	SUMF1	Multiple sulfatase deficiency	607939	AR	Ikeda et al,[77] 1998

(*continued on next page*)

Table 2
(*continued*)

Conditions	Gene	Disorder	OMIM	MOI	References
Immunodeficiencies	*FAS*	FAS-related autoimmune lymphoproliferative syndrome	601859	AD	Rudman Spergel et al,[78] 2013
	22q11.2	22q11.2 deletion syndrome	611867	AD	McDonald-McGinn et al,[79] 2024
	CYBB	CYBB-related chronic granulomatous disease	306400	XL	de Boer et al,[80] 1998
	WAS	Wiskott-Aldrich syndrome (See WAS-Related Disorders.)	300392	XL	Albert et al,[81] 2010
	CD3E	Severe combined immunodeficiency	615615	AR	de Saint Basile et al,[82] 2004
	IL2RG	X-linked severe combined immunodeficiency	300400	XL	Bode et al,[83] 2015
	IL7RA	Severe Combined Immunodeficiency	146661	AR	Puel et al,[84] 2000
	RAG1 and 2	Severe Combined Immunodeficiency	601457	AR	Schwarz et al,[85] 1996
Other	*ADAMTS13*	Hereditary thrombotic-thrombocytopenic purpura (Upshaw Schulman syndrome)	274150	AR	Hassenpflug et al,[86] 2018
	TCN2	Transcobalamin II deficiency (& other errors of vitamin B12 metabolism)	275350	AR	Unal et al,[87] 2014; Ünal et al,[88] 2019

Abbreviations: AD, autosomal dominant; AR, autosomal recessive; MOI, mode of inheritance; XL, X-linked.

is characterized by recurrent infections, oculocutaneous albinism, bleeding diathesis, neutropenia and pulmonary fibrosis. The *AP3B1* encoded protein is involved in protein trafficking to various vesicles from the Golgi apparatus. In an animal model, *Ap3b1* deficiency resulted in impaired virus control caused by a moderate defect in CTL cytotoxicity and demonstrated all HLH features. However, the disease was transient, and animal subjects were recovered with full control of the infection.[21] Therefore, HPS2 is likely to confer a risk for HLH, but that is lower than in GS2 or CHS, because of a milder defect in cytotoxicity.

RHOG

RhoG is a small GTPase that assists MUNC13-4 in docking to the cell membrane, and subsequent membrane fusion and release of cytotoxic granule content. In 2021, the first case of a 4-month-old infant with severe HLH was found to carry compound heterozygous mutations in *RHOG*.[22] In this patient, RhoG deficiency abrogated CTL and NK cell cytotoxicity. Interestingly, imaging studies of this patient's long bones revealed radiolucent bone lesions, increased sclerosis, and cupping on distal metaphyses. Whether these abnormalities are related to RhoG deficiency remains unknown. As no other RhoG-deficient patient has been reported, the full spectrum of clinical manifestations and effects of RhoG deficiency need to be further elucidated.

LYMPHOPROLIFERATIVE DISORDERS

SH2D1A (XLP1)

SH2D1A is the gene mutated in XLP1, a rare X-linked lymphoproliferative disorder associated with Epstein-Barr virus (EBV)-induced HLH. It has 3 classic manifestations, including EBV-HLH, lymphoma (most often of B-cell origin), and hypo- or dysgammaglobulinemia. Additional features include CNS vasculitis, pulmonary vasculitis and/or lymphomatoid granulomatosis, and aplastic anemia.[23] *SH2D1A* encodes the signaling lymphocytic activation molecule (SLAM)-Associated Protein (SAP), which recruits the tyrosine kinase FYN to the SLAM family of receptors. It also prevents the binding of inhibitory phosphatases. In so doing, SAP enables the generation of downstream signals critical for CD4 T cell cytokine production and helps to B cells, CD8 T, and NK cell cytotoxicity, CD8 T cell restimulation-induced cell death (RICD), and iNKT cell development. Based on emerging data, it is proposed that XLP1-associated EBV-HLH is due to poor B cell clearance, reduced T cell RICD, and preferential T cell production of proinflammatory cytokines, which together lead to dysregulated B and T cell proliferation, activation, and accumulation, and ultimately, excessive macrophage activation.

XIAP (XLP2)

XIAP is the causal gene for XLP2, a disorder resembling XLP1 that was first reported in 2006. While XLP2 and XLP1 share susceptibility to developing hyperinflammation, males with XLP2 are more likely to develop HLH even in the absence of prior EBV infection. They are also at risk of developing inflammatory colitis and hepatitis, which can progress to full-blown liver failure. Additional manifestations include splenomegaly, uveitis, arthritis, and other autoimmune disorders.[24] *XIAP* encodes the X-linked inhibitor of apoptosis, which affects the function of caspases-3, 7, and 9. Accordingly, loss of XIAP expression is associated with increased cell death, a property proposed to limit the expansion of T cells following viral infections in XLP2.[25] XIAP is also involved in regulating NOD1/2 signaling, NLRP3 inflammasome activation, and elimination of intracellular bacteria.[26,27] Altogether, XIAP loss results in dysregulation of innate and adaptive immune responses, which together contribute to HLH and other disease manifestations.

PRIMARY IMMUNE DISORDERS AND METABOLIC DISORDERS

HLH can complicate primary immunodeficiency disorders (PIDs) other than those just described. In these disorders, HLH can be initiated due to ineffective immune responses that cannot remove an infectious trigger (e.g., EBV), yet lead to hypercytokinemia and inappropriate immune system activation. Some of the metabolic disorders can also be the basis for genetic predisposition to HLH (**Table 2**). Mechanisms are complex and not well described, but deposits accumulating in macrophages are perceived as the most relevant for many diseases, as they may alter their function and lead to HLH.[28] HLH can be the first manifestation of PID or metabolic disorder, so those diseases should be considered in the differential diagnosis of HLH.[29]

APPROACHES TO MAKING A GENETIC DIAGNOSIS

Establishing a genetic diagnosis of fHLH typically involves a tiered approach, starting with the most efficient and cost-effective methods and progressing to more comprehensive tests if needed. The specific strategy may vary depending on the clinical setting and available resources. For patients with a high suspicion of fHLH based on clinical presentation or family history, initial testing includes sequencing of genes associated with fHLH, such as *PRF1, UNC13D, STX11,* and *STXBP2*. However, single-gene tests are limited in scope and sensitivity since they may not identify mutations in other genes associated with fHLH. Accordingly, they are generally reserved for families with a known mutation in a particular HLH-related gene, and/or a patient with a positive functional assay that points toward a mutation in a specific protein and/or pathway. For example, patients with low or absent expression of perforin, SAP, or XIAP protein as assessed by flow cytometry or western blotting,[30] yet normal lymphocyte degranulation (i.e., normal CD107a upregulation) represent good candidates to undergo single gene testing for mutations affecting *PRF1*, *SH2D1A,* or *XIAP,* respectively. Alternatively, individuals with normal perforin, SAP, and XIAP expression, yet reduced CD107a upregulation represent candidates for whom the *UNC13D*, *STX11,* and *STXBP2* genes should be evaluated. With a positive family history and certain ethnic backgrounds, targeted gene/mutation detection may be a good first step, such as examining for the *PRF1-c.50del T* in African American[31] and *UNC13D-c.118-308C>T* and *c.754-1G>C* in Korean[32] individuals due to founder effects, especially when there are limited resources for more comprehensive testing.

If no pathogenic variants are identified in the fHLH genes, broader testing using next-generation sequencing (NGS) panels that cover other immune dysregulation genes may be warranted. NGS panels are the preferred test design for fHLH as they allow simultaneous analysis of multiple HLH-associated genes, improving diagnostic yield.[33] These panels typically have high sensitivity for detecting point mutations and small insertions and deletions of a few nucleotides within the genes being targeted. However, their ability to detect noncoding, structural, and larger deletions or duplications may be limited. For example, in FHL3, the *UNC13D* gene can be inactivated via deep intronic and complex inversion mutations[34] that are not detected by NGS panel. They require specially designed tests tailored to each structural variation and/or whole genome sequencing.

Finally, whole-exome sequencing (WES) or whole-genome sequencing (WGS)[35] may be considered in cases where conventional testing does not yield a diagnosis. WES captures almost all coding regions of the genome, and WGS adds the ability to detect noncoding and structural variations and is considered the most sensitive test at the present time. These tests are particularly useful for identifying rare variants in atypical

presentations of HLH or for cases where other genetic tests fail to provide a diagnosis. While these tests offer higher diagnostic accuracy, they are costly, have longer turnaround times, and may yield incidental findings that require additional genetic counseling and follow-up evaluation. In general, clinicians should consider starting with an HLH gene panel and reserve WES or WGS for patients for whom gene panel testing is negative or patients with atypical and/or more complex clinical phenotypes.

Clinical genetic tests for HLH are designed for immediate patient care and provide clinically validated, actionable results on known HLH-related genes with well-established disease associations. These tests undergo rigorous validation[36] to ensure accuracy, sensitivity, and specificity for diagnostic purposes and are commonly performed in a clinical laboratory accredited by regulatory agencies. Clinical tests provide a definitive diagnosis that providers can use to make treatment decisions, but they may be limited in scope as they target only specific genetic variants. Research genetic assays, on the other hand, are exploratory and may involve WES, WGS, or specialized experimental techniques. These assays are used primarily to discover new genes or mutations linked to HLH and to study the underlying mechanisms of the disease. Both clinical and research assays may yield variants of uncertain significance (VUS) that require further study and cannot be used for immediate clinical decision-making.[37] Consequently, research assays may not provide clear diagnostic results but can contribute to a better understanding of the biologic basis of HLH and facilitate development of rational and more effective therapies.

In summary, establishing a genetic diagnosis for fHLH is a complex process requiring careful selection of tests based on clinical presentation, cost considerations, and available resources. Genetic testing options range from single-gene tests to NGS panels, WES, and WGS, each with its pros and cons. Accurate diagnosis relies not only on choosing the right test but also on utilizing various databases and genetic resources for interpretation (**Table 3**). Genetic counseling is essential for patients undergoing genetic testing for fHLH, as the implications of test results can significantly impact family planning and treatment decisions. Counselors help patients and families understand the genetic basis of fHLH, the implications of a positive or negative result, and the possibility of identifying VUS that may not be immediately actionable. Additionally, genetic counselors assist with interpreting the likelihood of fHLH recurrence in families and discuss options for prenatal or preimplantation genetic testing if desired. Genetic counseling also provides emotional support, as fHLH diagnosis can be overwhelming due to its severe prognosis and intensive treatment requirements. In summary, genetic counseling is a crucial aspect of this process, providing patients and families with the information and support they need to navigate the diagnosis and its implications. Together, these approaches and resources enable more precise and personalized care for patients with fHLH, improving clinical outcomes and quality of life for affected families.

CURRENT UNANSWERED QUESTIONS

Disease Classification

As stated above, the identification of fHLH is of paramount importance in guiding decisions surrounding treatment and family counseling. However, over the last 20 years, the distinction between fHLH and sHLH has become less distinct, making treatment choices even more complex. fHLH typically affects young children. However, late-onset and even adult-onset cases have been reported. In these patients clinical manifestations may be atypical or incomplete, leading to late recognition and fatal outcomes.[38] Most patients with late onset and/or milder phenotypes harbor hypomorphic

Table 3
Genetic_Genomic resources

Catergory	Resource Name	Website	References
Public population database	gnomAD	gnomAD browser (https://gnomad.broadinstitute.org/	Gudmundsson et al,[89] 2021
	NHLBI's Trans-Omics for Precision Medicine (TOPMed)-BRAVO	The NCBI dbGaP database of genotypes and phenotype(https://www.ncbi.nlm.nih.gov/gap/)	Taliun et al,[90] 2021
	The Geisinger Healthcare System DiscovEHR dataset	https://www.geisinger.org/precision-health/mycode/discovehr-project	Dewey et al,[91] 2016
	1000 Genome Project	http://www.1000genomes.org	Fairly et al,[92] 2019
	TCGA	https://www.cancer.gov/ccg/research/genome-sequencing/tcga	Gao et al,[93] 2019
Public disease database	ClinVar	https://www.ncbi.nlm.nih.gov/clinvar/	Landrum et al,[94] 2014
	HGMD	https://digitalinsights.qiagen.com/products-overview/clinical-insights-portfolio/human-gene-mutation-database	Stenson et al,[95] 2020
Prediction Tools for variant effects	Polyphen	http://genetics.bwh.harvard.edu/pph2/	Adzhubei et al,[96] 2010
	SIFT	https://sift.bii.a-star.edu.sg/	Sim et al,[97] 2013
	PMUT	https://mmb.irbbarcelona.org/PMut	López-Ferrando et al,[98] 2017
	Mutation tester	https://www.mutationtaster.org/	Schwarz et al,[99] 2014
	Franklin	https://franklin.genoox.com/clinical-db/home	Gennox inc.
	Condel	https://bio.tools/CONDEL	González-Pérez et al,[100] 2011
	CADD	https://cadd.gs.washington.edu/	Kircher et al,[101] 2014
Literrature Search Engine	PubMed	https://pubmed.ncbi.nlm.nih.gov/	White et al,[102] 2020
	Google Scholar	https://scholar.google.com/	Halevi et al,[103] 2017
Disease/Gene reviews	OMIM	https://omim.org/	Hamosh et al,[104] 2021
	GeneReviews	https://www.ncbi.nlm.nih.gov/books/NBK1116/	Adam et al,[105] 2024
Test/laboratory Directory	GTR	https://www.ncbi.nlm.nih.gov/gtr/	Wendy et al,[106] 2013

Abbreviations: gnomAD, Genome Aggregation Database; GTR, Genetic Testing Registry; HGMD, Human Gene Mutation Database; OMIM, Online Mendelian Inheritance in Man; TCGA, The Cancer Genome Atlas Program.

mutations in the fHLH-related genes, allowing for a residual protein expression or function that may cope with infectious triggers for some time and lead to less severe manifestations. Further, association studies have found an increased occurrence of monoallelic mutations in the fHLH-related genes in individuals with lymphoma, autoimmune lymphoproliferative syndrome, multiple sclerosis, arthritis, and other rheumatologic disorders.[8] Based on these observations, partial cytotoxic impairments caused by monoallelic mutations have been suggested to predispose to cancer, autoimmunity and/or HLH.[39] Moreover, data from mouse models showed that accumulation of monoallelic mutations in fHLH-related genes impairs cytotoxicity, contributing to HLH, suggesting a polygenic inheritance of the disease.[40] However, the clinical impact of monoallelic mutations in the context of fHLH remains poorly understood. Intensive treatments including HSCT are not justified in all patients with HLH who harbor monoallelic mutations. Thus, treatment decisions should be considered on a case-by-case basis in the context of each patient's clinical and laboratory findings.

Altogether, HLH can be regarded as a continuum of risk that is determined by different degrees of genetic predispositions and environmental factors. In this view, fHLH due to disruptive biallelic mutations and sHLH without overt mutations represent only the extremes of the spectrum. Individuals carrying hypomorphic biallelic or even monoallelic mutations in fHLH-related genes may require stronger environmental triggers to exceed the threshold of HLH development.[39] Adding further complexity, the advent of NGS has greatly expanded the spectrum of genes and genetic variants predisposing to HLH through a variety of immunological mechanisms (see **Table 2**).[41,42]

Genetic Variant Classification

A real challenge of NGS lies in evaluating the large number of detected variants that then must be filtered and evaluated for pathogenicity. Thanks to disease and population databases and computational prediction tools (see **Table 3**), variants can be assessed and classified into pathogenicity groups according to guidelines put forth by the Association for Clinical Genetic Science and the American College of Medical Genetics and Genomics.[37] Importantly, genetic testing may not identify the causative genetic variant in all fHLH patients. Further, the presence of a variant of unknown clinical significance (VUS), which represents the major percentage of variants found with high-throughput sequencing methods, can place both the patient and referring physician in a difficult position. In fact, of the approximately 2 million variants deposited in ClinVar, an archive of genetic variants, 36% are defined as VUS, and 5% have conflicting interpretations of pathogenicity while only 15% are pathogenic or likely pathogenic. This predominance of VUS reflects genetic results for which there are generally limited to no data available to verify their pathogenicity. Accordingly, these are not commonly used for making clinical treatment decisions. As one example of a challenging variant, *PRF1*-A91 V is reported to be one of the most common hypomorphic variants in patients with fHLH, but its role in the development of HLH is highly controversial. Within ClinVar, it has been interpreted as either benign, likely benign, pathogenic, or as a VUS by different laboratories. *PRF1*-A91V-associated clinical phenotypes in individuals with heterozygous, homozygous, and compound heterozygous with other *PRF1* mutations are highly variable, from asymptomatic to fatal cases.[43] This variant is present in 4.6% to 9.4% of the Caucasian population and has been associated with immune system activation, inflammation, and risk for autoimmunity and cancer.[44] Although the *PRF1*-A91 V mutation has been shown to impair perforin maturation to the active form,[45] further studies are necessary to fully assess the impact of the *PRF1*-A91 V variant. At present time, assays of protein expression are available for genes associated with fHLH, but they

are restricted to *PRF1, SH2D1A,* and *XIAP*. However, great effort is being invested in developing multiplexed, high-throughput functional assays for all types of genetic variants to interrogate their pathogenicity. Over time, the collaboration between clinicians and research scientists should improve our understanding of variants with conflicting interpretations of pathogenicity and pave the way toward a definitive genetic diagnosis for patients with fHLH and all genetic disorders.[46]

Genotype-Phenotype Associations

Despite genetic heterogeneity, similar clinical manifestations are common to the different genetic types of fHLH, making it difficult to discriminate the specific genetic defect at disease onset. However, some peculiar clinical features have been described. CNS involvement may be more common in individuals with *UNC13D*-fHLH than those with *PRF1*-fHLH.[7] Approximately one-third of *STXBP2*-fHLH individuals have additional clinical findings including colitis, bleeding disorders, and hypogammaglobulinemia.[15] Individuals with *STX11*-fHLH appear to have a later onset of manifestations and a milder disease course than those with *PRF1*-fHLH and *UNC13D*-fHLH.[9,10] Although the age of onset in individuals with STXBP2-fHLH tends to be later than in individuals with *PRF1*-fHLH or *UNC13D*-fHLH, the age of onset is variable even within the same family.[15] At the variant level, patients with biallelic loss-of-function mutations in *PRF1, STXBP2,* and *UNC13D* have a significantly younger onset age than patients with hypomorphic mutations.[38]

DISCUSSION AND FUTURE CONSIDERATIONS

Advances in, and application of, germline genomic testing have significantly contributed to our understanding of HLH, particularly in identifying the genes and genetic mutations[47] associated with the familial forms of the disease. Timely testing of the relevant genes has allowed for accurate diagnosis and management of HLH with chemotherapy, immunosuppressive therapy, and/or cytokine-directed therapy, as well as allogeneic hematopoietic stem cell transplantation. The implementation of comprehensive genetic panels has allowed clinicians to screen a broader range of genes associated with HLH and related immune system disorders, confirming diagnoses for symptomatic patients and identifying asymptomatic patients within families, a factor crucial for early diagnosis, close monitoring, and possible preemptive transplantation.

Nevertheless, while current genetic testing approaches provide valuable insights and have enhanced diagnostic capabilities, several considerations must be addressed to further improve upon current patient outcomes. First, it is essential to establish standardized guidelines for genetic testing for fHLH. This includes determining the most effective gene panels and the methods needed to assess the functional impacts of VUS. Second, the integration of genetic counseling into the diagnostic process will be vital to help families understand the implications of test results, including inheritance patterns and the potential risks for other family members. Moreover, the psychological impact of genetic testing and the stigma associated with genetic diseases must not be overlooked. Educational resources and support systems must be developed to assist families in coping with the implications of an fHLH diagnosis. Third, it is important to consider the need for ongoing research into the genotype-phenotype correlations in fHLH and fHLH mimics. Understanding how specific genetic mutations influence clinical presentations and severity of disease can provide valuable prognostic information and assist in tailoring individual treatment plans. Similarly, exploring the role of epigenetic factors and environmental triggers, such as EBV or other viral infections, could shed light on the complexities of fHLH pathophysiology. As the field advances, the

application of NGS testing can further enhance the speed and accuracy of testing, potentially enabling real-time analysis of emerging candidate genes. However, access to these technologies is fragmented with many regions of the world unable to obtain timely genetic testing and interpretation of results. Thus, a fourth consideration is how best to make genetic testing available to patients with fHLH around the world, including those in resource-limited settings. Finally, there must be a continued collaboration among researchers, clinicians, and patients to drive future innovations in diagnostic approaches and therapeutic management of fHLH. The integration of artificial intelligence for data analysis and interpretation could play a pivotal role in explaining complex genetic interactions, ultimately leading to the development of targeted therapies that improve patient outcomes. In summary, while the current state of genetic knowledge and testing for fHLH and related disorders of the immune system have improved our understanding and management of these disorders, ongoing advancements, collaborative efforts, and a focus on individualized patient care are essential for shaping the future landscape of genetic testing and treatment with the overall goal to improve patient outcomes.

CLINICS CARE POINTS

- The diagnosis of autosomal recessive inherited fHLH can be established by the identification of biallelic loss-of-function pathogenic variants in one of 4 genes: *PRF1*, *STX11*, *STXBP2*, or *UNC13D*.
- The finding of variants of uncertain significance (VUS) in one of these 4 genes does not establish or rule out the diagnosis of fHLH. Correlation with clinical and functional data is needed to support a possible diagnosis of fHLH.
- Once the fHLH-causing pathogenic variants have been identified in an affected individual, carrier testing of at-risk relatives becomes possible, as does prenatal and preimplantation genetic testing.
- For matched related donor HSCT, potential donors should be tested for the presence of the family-specific pathogenic variants to ensure that only individuals without biallelic fHLH-causing pathogenic variants are chosen as donors.
- Genetic testing for fHLH should be carried out in a clinical laboratory, and pregenetic and postgenetic counseling is highly recommended.

DISCLOSURE

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