Effective Immunological Guidance of Genetic Analyses Including Exome Sequencing in Patients Evaluated for Hemophagocytic Lymphohistiocytosis

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the HLH study of the GPOH

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Abstract

We report our experience in using flow cytometry-based immunological screening prospectively as a decision tool for the use of genetic studies in the diagnostic approach to patients with hemophagocytic lymphohistiocytosis (HLH). We restricted genetic analysis largely to patients with abnormal immunological screening, but included whole exome sequencing (WES) for those with normal findings upon Sanger sequencing. Among 290 children with suspected HLH analyzed between 2010 and 2014 (including 17 affected, but asymptomatic siblings), 87/162 patients with “full” HLH and 79/111 patients with “incomplete/atypical” HLH had normal immunological screening results. In 10 patients, degranulation could not be tested. Among the 166 patients with normal screening, genetic analysis was not performed in 107 (all with uneventful follow-up), while 154 single gene tests by Sanger sequencing in the remaining 59 patients only identified a single atypical CHS patient. Flow cytometry correctly predicted all 29 patients with FHL-2, XLP1 or 2. Among 85 patients with defective NK degranulation (including 13 asymptomatic siblings), 70 were Sanger sequenced resulting in a genetic diagnosis in 55 (79%). Eight patients underwent WES, revealing mutations in two known and one unknown cytotoxicity genes and one metabolic disease. FHL3 was the most frequent genetic diagnosis. Immunological screening provided an excellent decision tool for the need and depth of genetic analysis of HLH patients and provided functionally relevant information for rapid patient classification, contributing to a significant reduction in the time from diagnosis to transplantation in recent years.

Keywords

Hemophagocytic lymphohistocytosis
diagnosis
flow cytometry
degranulation
whole exome sequencing

Abbreviations

CHS	Chediak-Higashi syndrome
CTL	cytotoxic T cells
FHL	familial hemophagocytic lymphohistiocytosis
GPOH	group of the Society for Pediatric Oncology and Hematology
GS2	Griscelli syndrome type 2
HLH	hemophagocytic lymphohistiocytosis
HPS2	Hermansky-Pudlak syndrome type 2
HSCT	hematopoietic stem cell transplantation
LCH	Langerhans cell histiocytosis
MAS-HLH	Macrophage-activation syndrome
NGS	next-generation sequencing
NK cell	natural killer cell
PID	primary immunodeficiency
WES	whole exome sequencing
XLP	X-linked lymphoproliferate syndrome

Sandra Ammann, Kai Lehmberg contributed equally to this work.

Electronic supplementary material

The online version of this article (https://doi.org/10.1007/s10875-017-0443-1) contains supplementary material, which is available to authorized users.

Introduction

Hemophagocytic lymphohistiocytosis (HLH) is a life-threatening disorder characterized by severe hyperinflammation. HLH is not a single disease, but a characteristic syndrome that may develop in the context of various underlying conditions [1]. Genetic diseases affecting lymphocyte cytotoxicity, in which HLH is the key manifestation, are summarized as familial HLH (FHL2–5). In other genetic conditions affecting lymphocyte cytotoxicity, HLH can be one manifestation of a more complex syndrome (Griscelli syndrome type II (GS2), Chediak-Higashi syndrome (CHS), Hermansky-Pudlak syndrome type II (HPS2)) [2]. Two additional primary immunodeficiencies, X-linked lymphoproliferative disease type I and II, are associated with a high risk of HLH, particularly following EBV infection [3]. HLH developing in any of

these genetic diseases is usually termed primary (1°) HLH [4, 5]. In addition, there are “secondary” (2°) or “sporadic” forms of HLH, frequently induced by infections, in the absence of a biallelic or hemizygous defect in a gene associated with 1°HLH. 2°HLH also occurs in malignant, autoinflammatory, or autoimmune diseases, frequently also triggered or enhanced by infections [6]. Finally, 2°HLH occurs in genetic diseases, in which HLH is a rare, but nevertheless associated manifestation. This includes primary immunodeficiencies (PID) [7] and metabolic diseases [6].

The distinction between 1° and 2°HLH has important clinical implications because most patients with 1°HLH require hematopoietic ~~hematopoietic~~ stem cell transplantation (HSCT) ~~for most patients with 1°HLH~~ [8, 9, 10, 11]. Rapid identification of 1°HLH patients is paramount to facilitate immediate initiation of HSCT preparation. We and others have previously demonstrated the high sensitivity and specificity of intracellular protein staining and NK-cell and cytotoxic T cell (CTL) degranulation testing for identifying patients with 1°HLH [12, 13, 14, 15]. However, to which extent these tests can be used in clinical practice to reduce genetic screening, to direct more extensive genetic analysis including exome sequencing, or to expedite diagnosis has not been explored. Moreover, a recent paper speculated that with increased use of high throughput sequencing, immunological tests might be fully abandoned [16]. Thus, the significance of immunological evaluation of HLH patients remains debated.

We prospectively analyzed whether immunological studies can be used as a decision tool for the need and extent of genetic studies in 290 children referred for suspected HLH to the German HLH reference centers. Immunological screening performed before targeted sequencing of HLH-associated genes led to substantial reduction of genetic investigations and provided direct validation of the functional relevance of genetic findings. It was also used to identify patients with indication for whole exome sequencing (WES) on the basis of clear functional abnormalities, which helped resolve a few additional cases. This clinical practice provided functionally relevant information for treatment intensity and decisions on HSCT in patients with suspected HLH within 72 h. It has contributed to a significant reduction in the time from HLH diagnosis to HSCT in patients with primary HLH.

Methods

The German HLH Study

The HLH study group of the Society for Pediatric Oncology and Hematology in Germany, Austria, and Switzerland (GPOH) is coordinated by two reference centers (Hamburg and Freiburg). About 80–90% of children with suspected HLH in German hospitals are reported. Children with macrophage activation syndrome (MAS) in autoimmune or autoinflammatory diseases are usually only reported following hematological consultation for exclusion of 1°HLH or specific treatment issues.

Patient Cohort

All patients below age 16 reported to the HLH study for suspected HLH between 01/2010 and 12/2014 were included. Twenty international patients, mainly from Turkey and Eastern Europe, were included into the analysis of the diagnostic algorithm, but excluded from epidemiological calculations. Informed consent was obtained according to the institutional review board approval (University of Freiburg ethics committee's protocol numbers 143/12 and 40/08).

Clinical Patient Classification

Patients were classified as follows: (1) patients fulfilling at least 5/8 clinical criteria for HLH ("full" HLH), (2a) patients with sufficient documentation to state that they fulfilled less than 5/8 criteria ("incomplete HLH"), (2b) patients with liver failure or inflammatory CNS disease which are no formal HLH criteria, but prompted evaluation for 1°HLH ("atypical HLH"), (3) patients without any HLH symptoms, referred because of an affected relative or albinism. Of these, we included only those with a final 1°HLH diagnosis ("asymptomatic genetic disease"), (4) patients with insufficient documentation to state whether they fulfilled 5/8 criteria ("incomplete clinical information"). This mostly concerned patients referred for immunological screening, for which "incomplete/atypical" HLH was a remote differential diagnosis. In these patients, the referring physicians had frequently not quantified all HLH criteria at the time of requesting immunological analysis, which did not allow judgment, whether at least 5/8 criteria were fulfilled. Once provided with the negative immunological results, other diagnoses were pursued and outcome of further lab tests and final patient diagnosis were not reported to the HLH study group, leading to incomplete documentation. Among 388 patients referred for immunological evaluation in the study period, 98 patients were in this category leaving 290 patients for analyses.

Immunological Studies and Genetic Analyses

Degranulation assays with “fresh” (uncultured) and “activated” (incubated with 100 U/ml IL-2 for 48–72 h) NK cells were performed as published using protocol 1 [12]. Tests were repeated if the day control did not yield normal results. Results were classified as normal (fresh assay > 10% or fresh assay < 10% and activated assay > 35%) or defective (fresh assay < 10% and activated assay < 35%) based on previously published receiver operating characteristic analyses. In patients with abnormal protein stains, genomic DNA was Sanger sequenced for mutations in *PRF1* (FHL-2), *SH2D1A* (XLP-1), or *BIRC4A* (XLP-2). Patients with abnormal or defective degranulation were analyzed in parallel for mutations in *UNC13D* (FHL-3), including selected intronic regions [17, 18], and *STXBP2* (FHL-5), followed by analysis of *STX11* [19]. In patients with albinism, hair morphology and blood smear abnormalities guided the order of sequencing of *RAB27A* (GS-2), *LYST* (CHS), and *AP3B1* (HPS2). In the course of the study, we changed our policy and sequenced *RAB27A* also in patients without these manifestations [20]. Primer sequences are available upon request (U. zur Stadt). Exome sequencing was performed in unresolved cases with defective fresh NK degranulation as described [21]. Selected variants were confirmed by Sanger sequencing.

Final Patient Classification

Genetic variants were considered disease-causing (1°HLH), if they occurred in a homozygous, compound heterozygous, or hemizygous X-linked form and were shown to be functionally relevant by protein expression and/or functional analysis and/or had been published as HLH-associated in another patient. For patients with “full” HLH, we obtained follow-up information on survival, relapse of HLH and final diagnostic classification by the treating physician. Patients with a definite diagnosis of rheumatic disease, malignancy, metabolic disease, or PID were classified as such independent of the time of follow-up. Patients with HLH only associated with infection or HLH of unclear cause were classified as 2°HLH, if they lacked relapse within a follow-up period of at least 6 months.

Time to Transplant Analysis

To test whether time from clinical (not genetic) diagnosis of HLH to HSCT had changed after the introduction of immunological screening, we compared the study period (2010–2014) to a previous 5-year period (2003–2008). For

this, a competing risk analysis was performed with death prior to HSCT as competing event, using R 3.1.0 (R Foundation for Statistical Computing, Vienna Austria) [22].

Patients of the study cohort (2010–2014) with a diagnosis of primary HLH who received an HSCT or died prior to HSCT were included in the analysis (n = 38). Patients consecutively diagnosed with primary HLH between 2003 and 2008 were used for comparison (n = 38). However, in this cohort, we only included patients with a genetic defect that was known at the time of diagnosis (leading to exclusion patients with a retrospective diagnosis of FHL5). Patients diagnosed in 2009 were not included as diagnostic procedures were gradually modified during that year. Patients from outside Germany, asymptomatic patients, and patients with XIAP deficiency were excluded in both groups.

Results

Patient Cohort and Clinical Classification

We analyzed 290 patients referred between January 2010 and December 2014. This included 162 patients with “full HLH” (Fig. 1) and 111 patients with “incomplete HLH” or atypical HLH (CNS inflammatory disease or liver failure) (Fig. 2). We included 17 patients with asymptomatic genetic disease.

Fig. 1

Flow diagram of diagnostic evaluation of patients presenting with “full” HLH. Details on individual sequencing decisions including WES are provided in the text. *1* Perforin A91V mutations were not considered pathogenic. *2* Patient was sequenced because of pigmentation abnormalities. *3* WES was restricted to patients with fresh NK degranulation < 5%

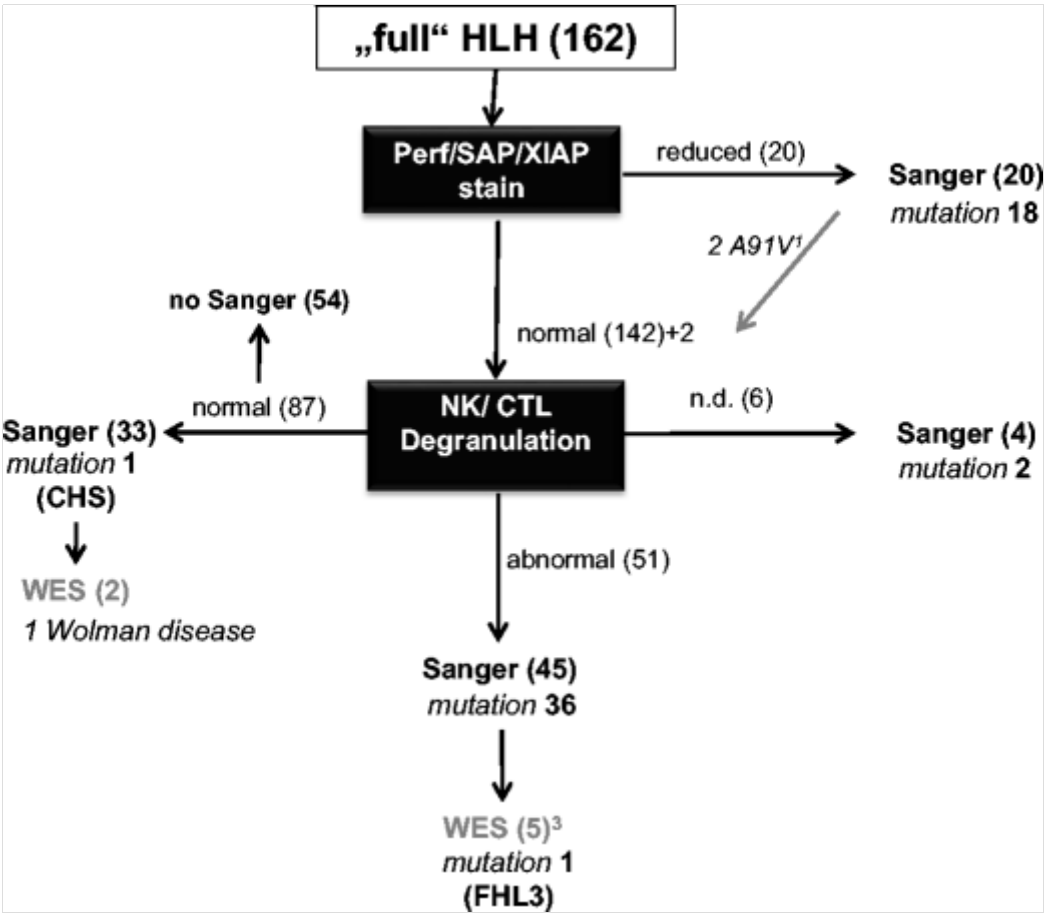
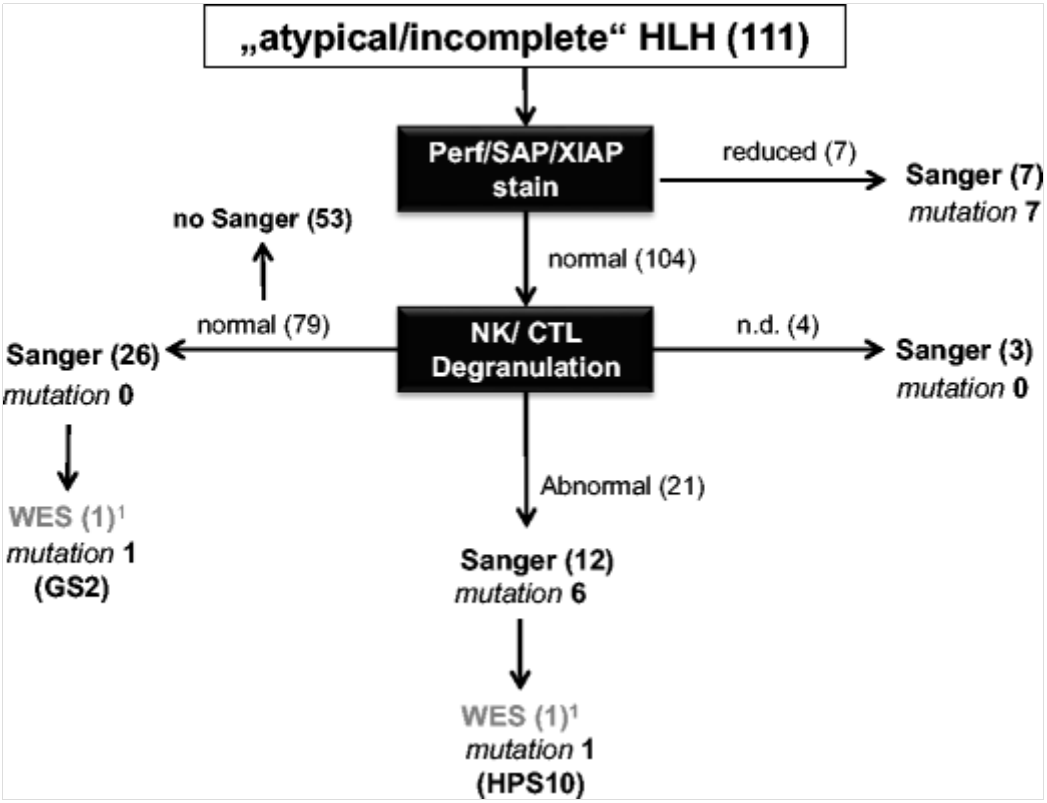


Fig. 2

Flow diagram of diagnostic evaluation of patients presenting with “incomplete/atypical” HLH. Details on individual sequencing decisions including WES are provided in the text. *1* WES was restricted to patients with fresh NK degranulation < 5%

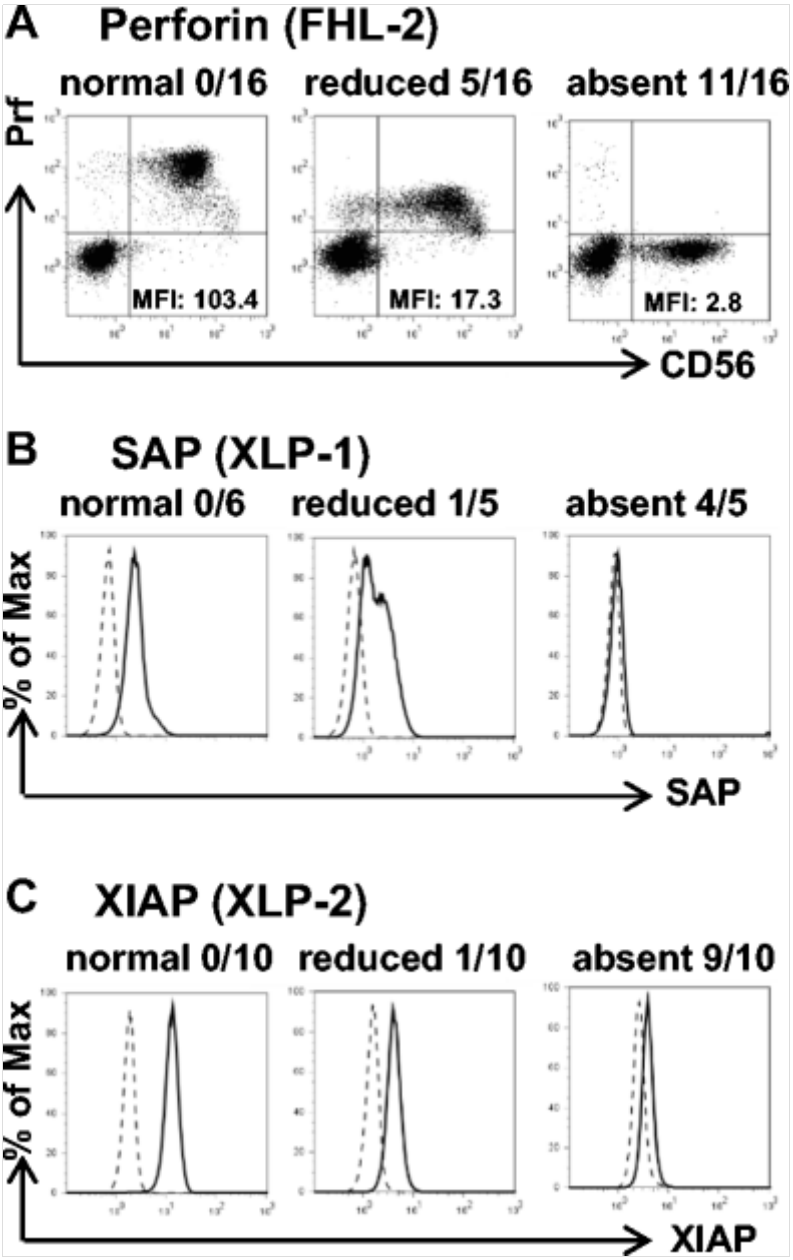


High Success Rate for FHL-2, XLP-1, and XLP-2 by Flow Cytometry Screening

All patients were screened by flow cytometry for perforin expression and male patients also for SAP or XIAP expression. Seven patients had reduced (Fig. 3, middle panels) protein expression and 24 patients had absent protein expression (Fig. 3, right panels). Disease-causing biallelic or hemizygous mutations were detected in all of them, except for 2 who had heterozygous perforin A91V mutations. Since normal protein expression has been reported in patients with SAP missense mutations, patients with high clinical suspicion for XLP were sequenced despite normal protein expression. However, in the present cohort, none of the patients with a final diagnosis of FHL-2, XLP-1, or XLP-2 had normal protein expression (Fig. 3, left panels).

Fig. ~~3~~4

Degranulation assays can reduce genetic investigations in patients with “full” HLH. NK degranulation was analyzed in 138 patients with “full” HLH and normal protein stainings. Left panel: fresh NK cell degranulation expressed as delta CD107a. Shaded gray: normal values; dotted line: cut-off below which genetic disease is likely[12]. Right panel: results of genetic analysis in the indicated cohorts grouped by fresh NK degranulation results and the results of stimulated NK degranulation This is Figure legend number 4. Sorry it is mixed up.



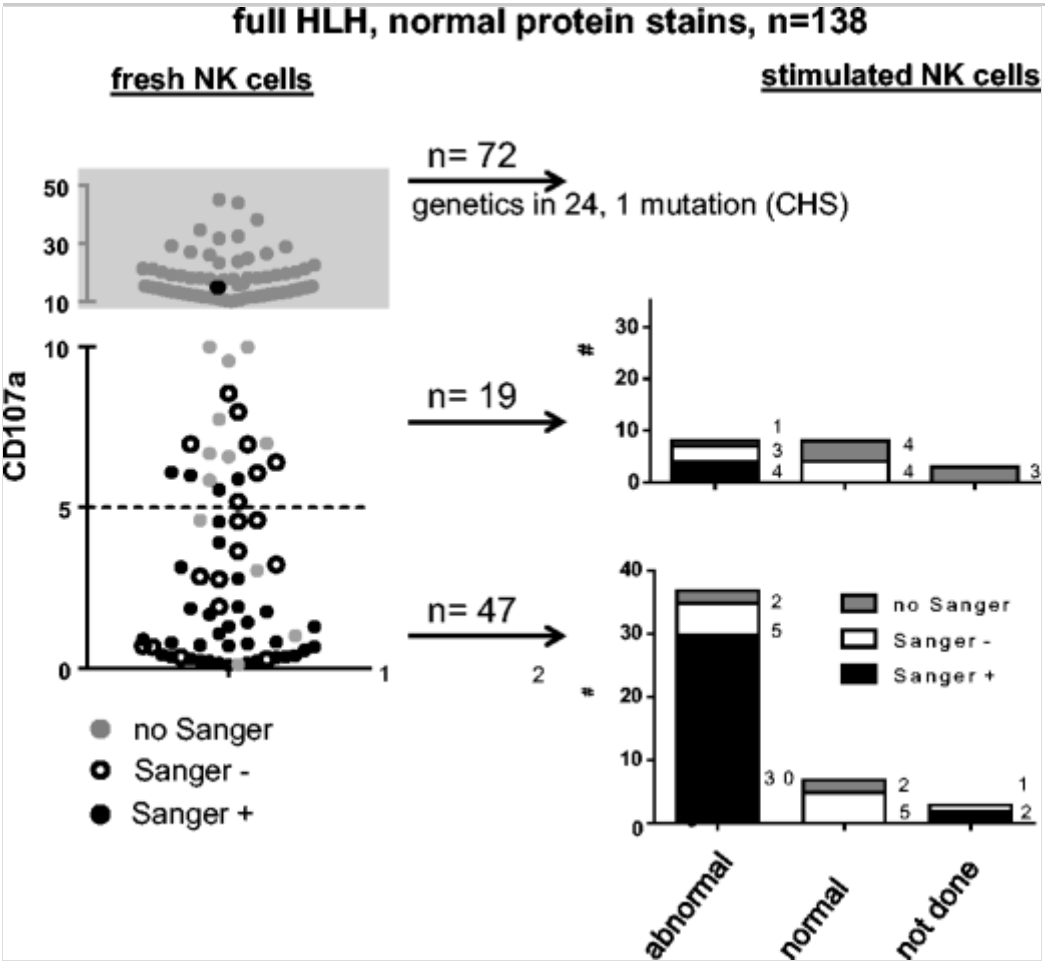
Effective Functional Screening for Genetic Degranulation Deficiency in Patients with "Full" HLH

Sixty-six patients with "full" HLH (41%) had fresh NK degranulation < 10%. In 60 of these, we performed additional degranulation testing of PHA/IL-2 stimulated NK cells, while lack of material did not allow further tests in 6 patients (Fig. 2). Among the 47 patients with 0–5% fresh NK degranulation, analysis of stimulated NK cells led to classification as "defective degranulation" in 39 (abnormal + not done). All but 2 of these patients (with a diagnosis of LCH and anaplastic lymphoma) were sequenced, leading to a genetic diagnosis in 32/37 patients. Among the 19 patients with 5–10% fresh NK degranulation (Fig. 4), 11 were classified as "defective degranulation" (abnormal + not done), 7 of these were sequenced, and mutations were found in 4. Four patients were not sequenced because of a diagnosis of SCID (1),

lymphoma (2), and lack of material (1). Overall, Sanger sequencing revealed disease-causing mutations in 34/44 patients with defective degranulation.

Fig. 43

Effective immunological screening by intracellular flow cytometry. **a** Perforin expression analysis in CD56+ NK cells. **b** Overlay of the fluorsecence signal of CD3+ cells stained with anti-SAP (solid line) or an isotype control (scattered line). **c** Overlay of the fluorsecence signal of CD3+ cells stained with anti-XIAP (solid line) or an isotype control (scattered line). **a**–**c** Sample plots for patients with normal, reduced, or absent protein expression. The relative number of patients showing the indicated pattern among all patients with the respective molecular diagnosis is shown This is figure legend number 3. Mixed up



Seventy-two of the 142 patients with “full” HLH and normal protein expression (51%) also had normal fresh NK degranulation, while this test was not performed in 6 patients (Fig. 1, Fig. 4). In one patient, sequencing was performed because of albinism and pathologic hair pigment distribution revealed a diagnosis of CHS (details below). Among the other 71 patients, no

genetic analysis was performed in 48, while 70 genes were sequenced in 23 patients because of the request of the referring physician. The monoallelic A91V perforin mutation was detected in one patient with Langerhans cell histiocytosis and EBV infection, and in one patient with genetically confirmed osteopetrosis. All 71 patients were classified as 2° HLH or as lost to follow-up on the following basis: 7 patients died in the acute phase of HLH, 3 of them in the context of malignant disease, 2 with syndromic diseases, and 2 in the context of EBV infection with negative genetic results. One patient had CGD. Another 53 patients had a follow-up of > 6 months (median 2.4 years, range 6 months to 4 years) without relapse. The remaining 11 patients (3 with MAS) were lost to follow-up.

The 12 patients who were not sequenced and the 18 patients with negative genetic results were classified as 2° HLH or lost to follow-up on the following basis: 5 patients died, 3 from malignant disease, 1 from Wolman disease, and one from unknown cause (Sanger sequenced in all known HLH-associated genes). One patient had SCID. Twenty-two patients had no disease recurrence within a median of 1.6-year follow-up (range 0.7–4.9 years). One patient had a second HLH episode 2 years later but no mutation in known genes, while one patient was lost to follow-up.

The Use of Degranulation Assays for Rational Genetic Testing in Patients with “Incomplete/Atypical” HLH

In the group with “incomplete/atypical” HLH, 7 of 111 patients had reduced or absent expression of *PRF1* (1), *SH2D1A* (2), or *XIAP* (4) (Fig. 2). Fresh NK degranulation was analyzed in 100 of the remaining 104 patients, with a normal result in 79 (79%) (Fig. 2, Fig. 5). In 26 of these, 52 genetic tests were performed based on requests by the referring physicians. All were negative. Twenty-five patients had fresh NK degranulation between 5 and 10% (Fig. 5). In 7 of them including 1 with abnormal stimulated NK/degranulation, genetic analysis was performed, but no mutation was found. Among 20 patients with fresh NK degranulation between 0 and 5%, mutations were found in 6/8 patients with abnormal stimulated NK/CTL degranulation and 0/8 patients where this second test was normal (Fig. 5). Table 1 shows details of the patients with “incomplete/atypical” presentation in whom genetic testing revealed 1°HLH.

Fig. 5

Degranulation assays can reduce genetic investigations in patients with “incomplete/atypical” HLH. Patients with “incomplete/atypical” HLH and

normal protein stainings were analyzed for NK degranulation. Left panel: fresh NK cell degranulation results expressed as delta CD107a. Shaded gray: normal values; dotted line: cut-off below which genetic disease is likely[12]. Right panel: results of genetic analysis in the indicated cohorts grouped by fresh NK degranulation results and the results of stimulated NK degranulation results. Black and white indicate presence or absence of a genetic degranulation defect, gray: no genetic investigations performed

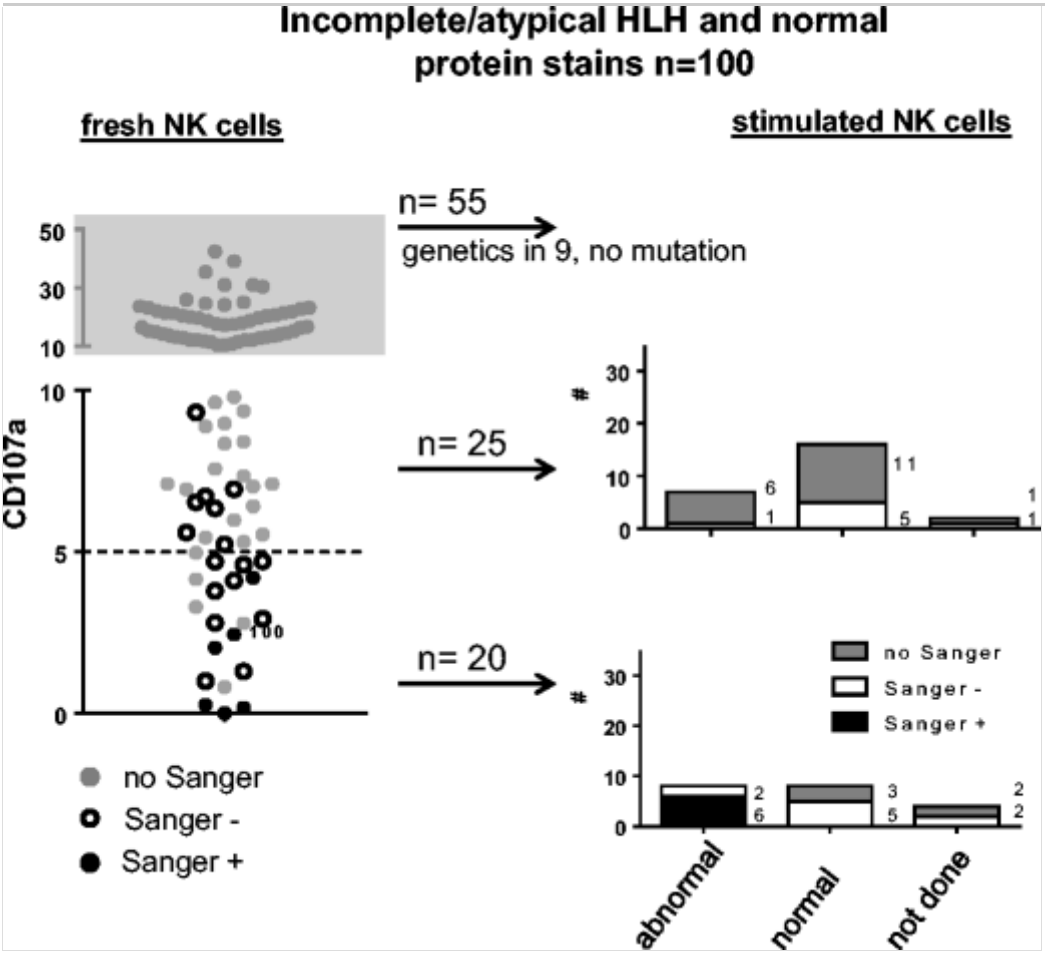


Table 1

Patients with “incomplete” or “atypical” HLH and positive genetic testing

Pat#	Final diagnosis	Clinical presentation	Mutation	Protein expression	Age at onset	Trig
3947	FHL-2	CNS only	homozygous c.208G > T	absent	10.4	–
3778	FHL-3	CNS and incomplete	homozygous c.1208C > T	n.d	1.0	–
2834	FHL-3	CNS and incomplete	Not reported by referring center	n.d	5.1	–

Pat#	Final diagnosis	Clinical presentation	Mutation	Protein expression	Age at onset	Tri
4389	FHL-5	incomplete	homozygous c.781C > T	n.d	0.4	–
2076	FHL-5	CNS and hepatitis	homozygous c.1247(−1) G > C	n.d	9.1	–
695	GS2	incomplete	homozygous c.428 T > C	n.d	4.0	EBV
1315	HPS2	incomplete	homozygous c.2041G > T	n.d	1.7	–
4385	XLP1	CNS and incomplete	hemizygous c.199G > T	absent	4.5	–
586	XLP1	incomplete	hemizygous c.79G > A	absent	7.8	unkr
2490	XLP2	incomplete	hemizygous c.728_749del22	absent	12.3	unkr
866	XLP2	incomplete	hemizygous c.997_1001DCAGAA	absent	14.0	–
904	XLP2	incomplete	hemizygous c.1141C > T	absent	7.2	EBV
1059	XLP2	incomplete	hemizygous c.1A > T	reduced	9.6	EBV

Exome Sequencing Uncovers Additional Patients with 1°HLH

Patients with defective fresh NK degranulation (< 5%), but no mutations in 1°HLH genes upon Sanger sequencing qualified for WES. In 12 of 20 patients fulfilling these criteria (Figs. 4 and 5), WES was not performed because there was insufficient material, the family denied consent or because the physician did not want to pursue this option because a plausible diagnosis such as malignancy or metabolic disease had been made (suppl. Tab. 2). Using WES, a genetic diagnosis could be established in four patients from consanguineous families (Table 2). In one patient with “full” HLH, we detected an unknown homozygous inversion of 1.65 MB involving the gene *UNC13D* with intronic breakpoints. This mutation had been missed in the conventional sequencing procedure based on amplification of single exons, which had rendered normal PCR products and sequences. Using WES, the breakpoints of this new inversion were assigned to intron 25 of *UNC13D*, 110 bp downstream of exon

25, and intron 3 of TTYH2. A Western blot analysis revealed complete absence of the Munc13–4 protein (Suppl. Fig. 1), confirming FHL-3. The second patient with "full" HLH had the homozygous mutation c.553delT resulting in a frame shift in the gene encoding lysosomal acid lipase (*LIPA*) and was diagnosed with Wolman disease, which is known to sometimes present with an HLH phenotype [23]. 1°HLH was also diagnosed in two patients with "incomplete/atypical" HLH. This included one patient with GS-2, in whom *RAB27A* had initially not been sequenced because he lacked albinism and hair abnormalities. The second patient carried a biallelic deletion in the gene encoding AP-3δ. This patient was given the novel diagnosis of HPS-10; details were reported elsewhere [21]. In four non-consanguineous patients with "full" HLH, analysis of WES data did so far not yield a plausible candidate gene.

Table 2

Patients analyzed by whole exome sequencing (WES)

Pat#	HLH	Fresh NK (%)	Stimulated NK/CTL	Con-sang	FH	Age at onset	Disease	
2920	Full	0.35	Abnormal	yes	yes	0.3	FHL-3	UNC13D:c.2 biallelic
1316	Full	0.67	n.d.	yes	no	0.5	?	?
1996	Full	2.77	Normal	no	no	0.1	?	?
1398	Full	3.22	Normal	yes	n.a.	0.2	LAL Def	LIPA:c.553de
2215	Full	0.7	Abnormal	no	yes	13	?	?
710	Full	4.6	Abnormal	no	n.a.	0.1	?	?
659	incompl	1.3	Abnormal	yes	no	3.1	HPS-10	AP3D1:c.356 biallelic
3434	incompl	4.6	Normal	yes	yes	10.0	GS-2	RAB27A:c.1

FH family history, *n.a.* not available, *n.d.* not done

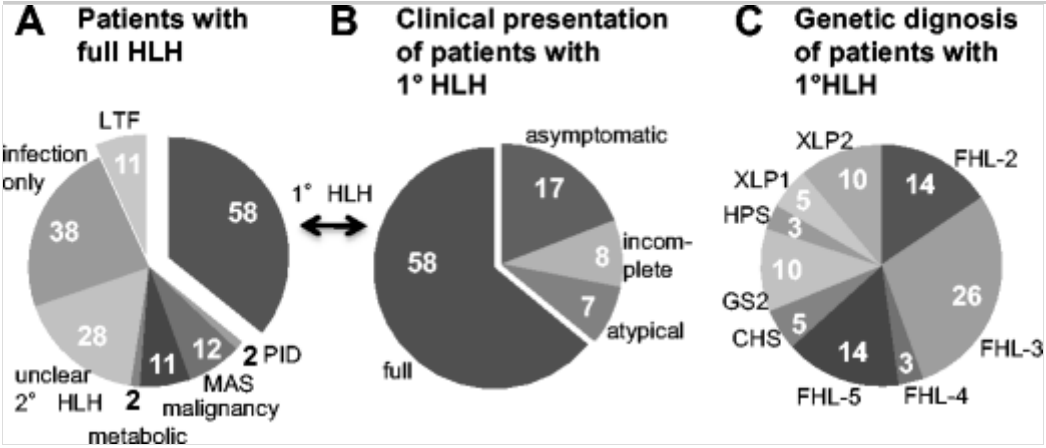
Distribution of Different Forms and Genetic Subtypes of 1° and 2°HLH in Germany

Overall, among the 290 patients referred for suspected HLH and sufficient information for final disease classification, 162 had "full" HLH. Of these, 58

patients (44 FHL2–5) had a final diagnosis of 1°HLH (35%), while 93 patients had 2°HLH (57%) (Fig. 6a). This included 12 patients with rheumatic disease, 11 with malignancy, one patient each with common γ-chain deficiency, X-linked chronic granulomatous disease and prolidase deficiency, and 38 patients in whom an infection was the only associated clinical feature. In 28 patients, no clear underlying condition was found. Eleven patients (8%) were lost to follow-up. Notably, 1°HLH was also diagnosed in 32 patients in the absence of "full" HLH (Fig. 6b). Of these, 15 (5 FHL) had "incomplete/atypical" HLH and 17 (6 FHL) were asymptomatic at the time of genetic diagnosis. This means that no more than 64% of patients with a genetic disease predisposing to HLH (80% of patients with FHL2–5) were actually diagnosed in a disease episode fulfilling the clinical HLH criteria. Among patients with 1°HLH (Fig. 6c), FHL represented 64% with predominance of FHL-3 (29%), followed by FHL-5 (17%) and FHL-2 (16%). Albinism syndromes were diagnosed in 20% of patients, while 16% had XLP. This distribution was similar if the analysis was restricted to the 81 patients referred from German hospitals (not shown).

Fig. 6

Final classification of patients with "full HLH" and clinical presentation and genetic diagnosis of patients with 1°HLH. Final classification of patients with "full" HLH. The absolute number of patients with a given diagnosis is indicated in the sections of the plot. *PID* primary immunodeficiency, *MAS* macrophage activation syndrome, *LTF* lost to follow-up. **b** Clinical presentation of the 91 patients with a final diagnosis of 1°HLH. **c** Genetic diagnosis of the patients with 1°HLH



Immunological Screening Contributes to More Rapid HSCT in Patients with 1°HLH

To test whether time from clinical diagnosis of HLH to HSCT had changed after the introduction of immunological screening, we compared the study period (2010–2014) to a previous 5-year period (2003–2008). Time from clinical diagnosis of acute HLH to HSCT was significantly shorter in the time period after introduction of the screening algorithm (median 130 versus 206 days, $p = 0.007$, corrected for death prior to HSCT as competing event) (Fig. 7).

Fig. 7

Expedited HSCT after introduction of immunological screening. The cumulative performance of HSCT over time after the clinical diagnosis of HLH is shown. The difference to 1.0 is attributed to deaths prior to HSCT. The dashed line represents patients of the study cohort diagnosed 2010–2014 as primary HLH using flow cytometry screening ($n = 38$). Patients of the comparison group diagnosed 2003–2008 before the introduction of the screening are shown as solid line ($n = 38$). Time to HSCT was shorter in the screened group (median 130 days) than in the unscreened group (median 206 days). The difference was significant ($p = 0.007$) in a competing risk analysis with death prior to HSCT as competing event. Among other factors, the screening is likely to have contributed to earlier performance of HSCT

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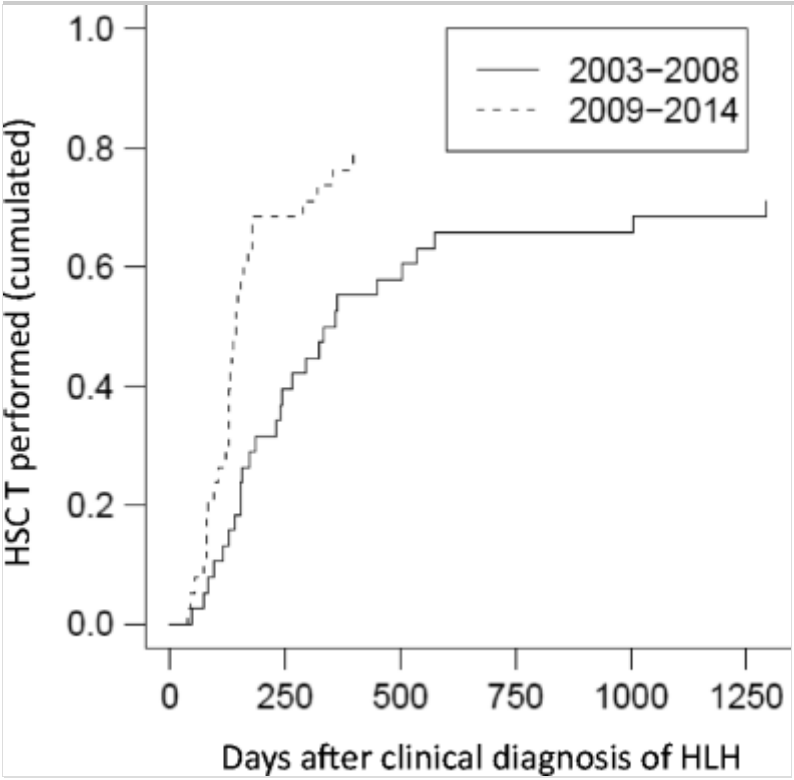

Discussion

In recent years, new diagnostic procedures have significantly changed the approach to patients with suspected HLH [24]. Identification of novel HLH-associated genes has improved possibilities to definitely diagnose 1°HLH [19, 25, 26]. More specific immunological tests facilitate rapid identification of patients in need of HSCT [12]. In addition, next-generation sequencing (NGS) allows more comprehensive genetic analyses while offering opportunities to identify novel genes predisposing to HLH [27]. Hence, diagnostic approaches are changing, and the most efficient algorithm remains controversial. Here, we prospectively evaluated the use of immunological screening as a decision tool for genetic studies including WES. The guiding principle was to provide the most rapid, reliable, and efficient basis for HSCT indication and other treatment decisions in patients with suspected HLH.

We performed three-step immunological screening with intracellular protein staining and fresh NK degranulation followed by activated NK degranulation in patients with abnormal results. We have previously shown that the fresh NK assay has excellent sensitivity (96%) and satisfying specificity (88%) [12]. Although both parameters are slightly inferior with activated NK degranulation [12], this second test improves the positive predictive value and was therefore added to guide our decision on sequencing in this study. We did not perform parallel cytotoxicity assays because of previously discussed limitations [12]. We did not sequence the perforin gene in patients with normal protein expression based on long-term experience in our and several other laboratories (K. Gilmour, Y. Bryceson, G. de Saint Basile, personal communication). In contrast, patients with a strong clinical suspicion for XLP were further analyzed despite normal protein expression. This included NKT cell quantification for XLP1 [28] and L-18 MDP stimulation for XLP2 [29], followed by genetic analysis in those patients, where high suspicion remained.

Among 162 patients with “full” HLH in this study, 58 patients had genetic disease. Our screening approach missed one patient with atypical CHS. It can be argued that the limited follow-up of the patients with “full” HLH (mean of 2.4 years) is not sufficient to exclude that some patients carry clinically relevant mutations and this remains a caveat. Among 111 patients with “atypical/incomplete” HLH, 15 had genetic disease. All of them were picked up by our screening approach. These findings confirm the excellent sensitivity of immunological screening but also show that it is not 100%.

It has been suggested that because of this limited sensitivity, all patients with

suspected HLH should undergo extensive sequencing. Such recommendations do not consider the limitations of genetic analyses. Thus, “hidden” intronic mutations or inversions or mutations in genes so far not associated with HLH but affecting the same pathway are not picked up with conventional genetic analysis. This leads to no better sensitivity of genetic compared to immunologic analysis. Moreover, extensive sequencing, in particular in the poorly defined cohort of patients with “incomplete” HLH, will generate many findings that are difficult to interpret in the absence of immunological data (such as monoallelic mutations). Restricting extensive sequencing efforts to patients with “full” HLH is also a questionable approach. In our study, 20% of patients with genetic disease did not present with this “classical” phenotype.

Overall, it is part of sound medical reasoning that any laboratory test (including genetics) has limitations and must be performed and interpreted in a clinical context. In light of the above considerations, immunological screening appears a rational approach to determine the need and the depth of sequencing in patients referred for evaluation for HLH. Earlier detection of primary HLH is a further strong argument for the use of the screening tests. We could demonstrate that the time from clinical diagnosis of HLH to HSCT was significantly shorter after the introduction of flow cytometric screening. There are certainly other factors that have accelerated the time to HSCT in recent years. However, since unambiguous abnormal screening results prompt initiation of HLA analysis and consecutive donor search within 72 h after diagnosis, we are convinced that our screening algorithm has contributed to this improvement.

Among those patients, where we performed genetic analysis, we detected biallelic or hemizygous mutations in 35% of patients with “full” HLH. This is similar to the Italian and Swedish cohorts [16, 30]. However, compared to the Italian cohort, FHL-2 was less frequent, while albinism syndromes and FHL-3 were more frequent. Notably, 33/91 patients (36%) with 1°HLH were not diagnosed in the context of a clinical episode fulfilling HLH criteria. This included 18 patients diagnosed in the absence of inflammatory manifestations and 15 patients diagnosed in the context of atypical/incomplete HLH. This further supports the repeatedly suggested revision of diagnostic criteria for HLH. The criteria should allow a diagnosis of 1°HLH in oligo- or asymptomatic patients in the context of immunological findings.

In patients with abnormal immunological screening, but no mutations found in the relevant genes and a suggestive clinical context, we extended our genetic

analysis to WES. We identified one patient with a novel inversion in *UNC13D*. Due to intronic breakpoints, this mutation had not been identified by Sanger sequencing. In one patient, absence of albinism was the basis for not sequencing *RAB27A*. We learned during this study that this should be included, as albinism is not necessarily present in GS-2 patients [20]. We also diagnosed one metabolic disease, Wolman disease [23]. Degranulation was low in that patient while it was normal in her sister with the same mutation. It remains unclear whether degranulation testing is useful in screening for this disease; however, we had another Wolman patient with reduced degranulation mentioned in a previous paper [12]. Only one patient was diagnosed with a new disease associated with impaired cytotoxicity, HPS10 [21]. Together with the findings of the Italian study [16], where 92% of patients with likely FHL received a genetic diagnosis, this supports the assumption that at least in European cohorts, most genes associated with impaired cytotoxicity that are linked to an HLH phenotype in humans have been discovered.

Our focus on functional screening may have led to underdiagnosis of monoallelic mutations. In the Italian study, 15% of patients with 2°HLH had monoallelic mutations in HLH-associated genes [16]. A similar frequency of monoallelic mutations was observed in a smaller Swedish cohort [30], but such monoallelic variants were not enriched compared to healthy individuals. Furthermore, parents or siblings with monoallelic mutations do not seem to have a predisposition to HLH, although a slightly increased risk of gynecological tumors has recently been demonstrated [31]. Particularly, there is little evidence to indicate that patients carrying monoallelic mutations have a transplant indication. This important question requires clear guidance. Based on the available evidence, it is our policy that in the absence of significant functional alterations [32], monoallelic mutations do not justify transplantation. This extends to the recommendation not to transplant after a first HLH episode in patients with homozygous A91V mutations [33, 34]. Equally, we consider carrier relatives as suitable stem cell donors and prefer a matched sibling with a monoallelic mutation without functional abnormalities to an unrelated wild-type donor.

Obviously, diagnostics of monogenic diseases is a field in transition. It has been hypothesized that NGS will soon allow rapid differentiation of 1°HLH from 2°HLH obviating the need for immunological tests [16]. We argue that this will depend on whether this approach will become rapid enough for a transplant decision. It will require fast workflows for individual samples that

are cost-effective. Moreover, without functional validation, the significance of some genetic alterations including monoallelic mutations remains unclear [35], in particular in the absence of comprehensive and well-curated mutation databases with clinical annotations. We therefore conclude that establishment of reference centers for the immunological evaluation of patients with suspected HLH is still a relevant investment into the future.

Acknowledgements

We thank the patients and their families as well as all physicians participating in the GPOH HLH study. We are grateful to the team of the CCI Advanced Diagnostic Unit for their excellent work. We thank Manuela Adao for excellent technical assistance and Marcus Tetzlaff for reliable documentation, and Martin Mynarek for performing competing event analysis with R (all Hamburg, Germany).
The following physicians contributed substantially to the HLH study of the GPOH (Germany, Austria, Switzerland):
Martina Ahlmann (Münster), Roland Ammann (Bern, Switzerland), Uta Behrends (Munich), Rita Beier (Essen), Horst von Bernuth (Berlin), Karin Beutel (Munich), Birgit Burkhardt (Münster), Gunnar Cario (Kiel), Carl-Friedrich Classen (Rostock), Matthias Dürken (Mannheim), Martin Ebinger (Tübingen), Johann Greil (Heidelberg), Ute Groß-Wieltsch (Stuttgart), Bernd Gruhn (Jena), Wolfgang Holter (Vienna, Austria), Patrick Hundsdörfer (Berlin), Ingrid Kühnle (Göttingen), Norbert Jorch (Bielefeld), Reinhard Kolb (Oldenburg), Jörn-Sven Kühl (Berlin), Britta Maecker (Hannover), Roland Meisel (Düsseldorf), Milen Minkov (Wien), Ingo Müller (Hamburg), Tim Niehuis (Krefeld), Jana Pachlopnik-Schmid (Zurich, Switzerland), Arnulf Pekrun (Bremen), Aram Prokop (Cologne), Johannes Rischewski (Luzern, Switzerland), Irene Schmid (Munich), Ansgar Schulz (Ulm), Paul-Gerhardt Schlegel (Würzburg), Michael Schündeln (Essen), Markus Seidel (Graz, Austria), Thorsten Simon (Köln), Jan Sörensen (Frankfurt), Martin Chada (Erlangen), Meinolf Suttorp (Dresden), Wilhelm Woessmann (Giessen).
Centers in Germany, unless otherwise specified.

Author's Contributions All authors gave input and approved the final version of the manuscript.

Sandra Ammann: Collected patient data, supervised routine immunological testing, performed additional confirmatory tests, drafted manuscript.

Kai Lehmberg: Coordinates the GPOH HLH study, collected patient data,

drafted manuscript.

Udo zur Stadt: Performed sequencing and provided genetic data.

Christian Klemann: Collected patient data.

Sebastian Bode: Collected patient data.

Carsten Speckmann: Collected patient data.

Gritta Janka: Initiated the GPOH study.

Katharina Wustrau: collected patient data for transplantation study.

Mirzokhid Rakhmanov: Coordinated diagnostic testing.

Ilka Fuchs: Coordinated diagnostic testing.

Hans Christian Hennies: Performed whole exome sequencing.

Stephan Ehl: designed the study, coordinates the GPOH HLH study, drafted the manuscript.

Funding This work was supported by grants from the DFG (EH 145/5-1 and SFB1160, TP1) and BMBF (01 EO 0803) to S.E., from the DFG (HE 3119/10-1) and the Köln Fortune Program of the Faculty of Medicine, University of Cologne to H.C.H.

Compliance with Ethical Standards

Informed consent was obtained according to the institutional review board approval (University of Freiburg ethics committee's protocol numbers 143/12 and 40/08).

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Conflicts of Interest SE has received consulting fees from UCB and Novartis, but not in relation to this study.

Electronic supplementary material

ESM 1

(PDF 67 kb).

ESM 2

(PPTX 91 kb).

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