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American Society of Hematology
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# Diagnostic Guidelines for Familial Hemophagocytic Lymphohistiocytosis Revisited

Tracking no: BLD-2024-025077R1

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**Abstract:**
Current HLH-2004-based diagnostic criteria for familial hemophagocytic lymphohistiocytosis (FHL) are based on expert opinion. Here we performed a case-control study to test and possibly improve these clinical criteria. We also developed two complementary expert opinion-based diagnostic strategies for FHL in patients with signs/symptoms suggestive of HLH, based on genetic and cellular cytotoxicity assays. The cases (n=366) were children <16 years with verified familial and/or genetic FHL (n=341) or Griscelli syndrome type 2 (GS2) (n=25); 276 from the HLH-94/HLH-2004 databases and 90 from the Italian HLH Registry. All fulfilled the HLH-94/HLH-2004 patient inclusion criteria. Controls were 374 children with systemic-onset juvenile idiopathic arthritis (sJIA) and 329+361 children in two cohorts with febrile infections that could be confused with HLH and sepsis, respectively. To provide complete data sets, multiple imputations were performed. The optimal model, based on the number of diagnostic criteria fulfilled from 17 variables studied, reveled almost similar diagnostic thresholds as the existing criteria, with accuracy 99.1% (sensitivity 97.1%; specificity 99.5%). Notably, assessment of the original HLH-2004 criteria revealed accuracy 97.4% (sensitivity 99.0%; specificity 97.1%). Since cellular cytotoxicity assays here constitute a separate diagnostic strategy, HLH-2004 criteria without NK-cell function was also studied which showed accuracy 99.0% (sensitivity 96.2%; specificity 99.5%). Thus, we conclude that the HLH-2004 criteria (without NK-cell function) have significant validity in their current form when tested against severe infections or sJIA. It is important to exclude underlying malignancies and atypical infections. In addition, complementary cellular and genetic diagnostic guidelines can facilitate necessary confirmation of clinical diagnosis.

**Conflict of interest:** COI declared - see note

**COI notes:** JIH, ES, RC, ARK, KL, ACH, and MBJ serve as consultants and/or in advisory boards for SOBI. MBJ also has research support from SOBI. RC also serves as consultant for Pfizer, Abbvie, Curbside Consults, Vindico Medical Education, Lilly, and unpaid consultant for APOLLO Therapeutics. ACH also serves as a speaker for Novartis. KK's work is funded by K23GM148827. AN is on the advisory board for JAZZ pharmaceuticals. SC has received consulting fees from Apollo and Simcha therapeutics, and speaker fees from SOBI and PracticePoint communications, JE, EB, IHM, MLC, FM, AR, YMT, MB and YTB have no conflicts to declare.

**Preprint server:** No;

**Author contributions and disclosures:** JIH, ACH, and MBJ planned the study, with JIH as principal investigator. JIH, EB, ACH, MLC, and ES provided data summaries on cases. FM, KFK, RQC, AR, and SWC, provided data summaries on controls. JE, IHM and MB performed statistical analyses. YTB and ES developed the format for the genetic and functional pathways. JIH, ACH, JE, ARK, KL, AN, YMT, MBJ, ES, and YTB analyzed data. JIH drafted the manuscript, assisted by JE and YTB, and it was reviewed and approved by all authors.

**Non-author contributions and disclosures:** No;

**Agreement to Share Publication-Related Data and Data Sharing Statement:** Data cannot be shared because this was not applied for in the ethical applications.

**Clinical trial registration information (if any):**

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Data cannot be shared because this was not applied for in the ethical applications.

**Short title:** Diagnostic guidelines for FHL revisited

**Counts:** Text: 4000 words. Abstract: 250 words. Tables: 5. Figures: 1. References: 50. Supplemental Tables: 6. Supplemental Figures: 2.

**Scientific category:** Clinical trials and observations

## KEY POINTS:

**Key point 1:** We present updated diagnostic criteria for FHL including revised clinical criteria and guidelines on genetic and cellular diagnostic assays.

**Key point 2:** HLH-2004 criteria (without NK-cell function) have significant validity (accuracy = 99%) tested against infections and systemic inflammation.

## ABSTRACT

Current HLH-2004-based diagnostic criteria for familial hemophagocytic lymphohistiocytosis (FHL) are based on expert opinion. Here we performed a case-control study to test and possibly improve these clinical criteria. We also developed two complementary expert opinion-based diagnostic strategies for FHL in patients with signs/symptoms suggestive of HLH, based on genetic and cellular cytotoxicity assays. The cases (n=366) were children <16 years with verified familial and/or genetic FHL (n=341) or Griscelli syndrome type 2 (GS2) (n=25); 276 from the HLH-94/HLH-2004 databases and 90 from the Italian HLH Registry. All fulfilled the HLH-94/HLH-2004 patient inclusion criteria. Controls were 374 children with systemic-onset juvenile idiopathic arthritis (sJIA) and 329+361 children in two cohorts with febrile infections that could be confused with HLH and sepsis, respectively. To provide complete data sets, multiple imputations were performed. The optimal model, based on the number of diagnostic criteria fulfilled from 17 variables studied, reveled almost similar diagnostic thresholds as the existing criteria, with accuracy 99.1% (sensitivity 97.1%; specificity 99.5%). Notably, assessment of the original HLH-2004 criteria revealed accuracy 97.4% (sensitivity 99.0%; specificity 97.1%). Since cellular cytotoxicity assays here constitute a separate diagnostic strategy, HLH-2004 criteria without NK-cell function was also studied which showed accuracy 99.0% (sensitivity 96.2%; specificity 99.5%). Thus, we conclude that the HLH-2004 criteria (without NK-cell function) have significant validity in their current form when tested against severe infections or sJIA. It is important to exclude underlying malignancies and atypical infections. In addition, complementary cellular and genetic diagnostic guidelines can facilitate necessary confirmation of clinical diagnosis.

## INTRODUCTION

Familial hemophagocytic lymphohistiocytosis (FHL) is a severe hyperinflammatory condition with accumulation of macrophages and lymphocytes in tissues[1-3]. This condition is caused by autosomal recessive variants in one of four genes FHL2-5, associated with *PRF1*, *UNC13D*, *STX11*, and *STXBP2*, respectively, all resulting in impaired lymphocyte cytotoxicity[4-9]. Typical manifestations include prolonged fever, splenomegaly, cytopenias, hypertriglyceridemia, hypofibrinogenemia, and hyperferritinemia[10-12]. The most frequent severe sequelae are neurological deficits associated with central nervous system (CNS) involvement[1-3,13,14].

Because of the high risk for death and CNS damage if adequate treatment is delayed, early diagnosis is crucial. In clinical practice, typically the first step is to identify the HLH syndrome and, secondly, confirm its underlying cause. Correspondingly, we here present an analysis testing HLH-associated clinical and laboratory criteria, and then guidelines for a genetic diagnosis, precise but time-consuming, and for a biological diagnosis, based on functional cellular assays[15]. Such assays of lymphocyte phenotype and function can rapidly provide vital insights in HLH patients awaiting or not subjected to genetic analysis, with genetic variants of unknown or unclear significance or altogether lacking variants in HLH-associated genes yet with suspicion of congenital HLH.

Currently, diagnosis of HLH is in most centers based on the HLH-2004 trial enrollment criteria[11], developed from the HLH-94 study criteria where 5/5 criteria (fever, splenomegaly, bicytopenia (hemoglobin <90g/L, platelets $<100x10^9$/L, neutrophils $<1.0x10^9$/L), hypertriglyceridemia (fasting triglycerides ≥2.0mmol/L) and/or hypofibrinogenemia (fibrinogen ≤1.5g/L), and hemophagocytosis) were required to be included, alternatively an affected sibling (*i.e.*, familial cases)[10]. In the HLH-2004 study, three new diagnostic criteria were added; ferritin ≥500µg/L, low/absent NK-cell activity, and soluble CD25 (sCD25) ≥2,400U/mL, and the level of fasting triglycerides was changed to ≥3.0mmol/L. Altogether 5/8 diagnostic criteria, an affected sibling, or biallelic variants in genes associated with FHL2-5 were criteria for enrollment (Table 1)[11].

We performed a case-control study aiming to both validate the current HLH-2004 criteria, based on expert opinion, and to determine if they improved by making up another set of diagnostic criteria or a diagnostic score. We analyzed a large cohort of children with verified molecular and/or familial diagnosis of FHL, or the closely related Griscelli syndrome type 2 (GS2) where variants in *RAB27A* result in impaired lymphocyte cytotoxicity[16]. These data were compared to three cohorts of control patients, *i.e.*, children with clinical presentations similar to FHL. We aimed to find a relevant balance between a predictive model which would optimize diagnostic criteria statistically, but also be reasonably easy to use clinically. For the genetic and cellular pathways, the recommendations presented are based on expert opinion.

## STUDY POPULATION AND METHODS

### FHL cases and controls

The cases (n=366) were children aged <16 years (according to the HLH-94 study age limit) with verified familial and/or molecular diagnosis of FHL (n=341) or GS2 (n=25); 82 from the HLH-94 database,[10] 194 from the HLH-2004 database,[11] and 90 from the Italian HLH Registry; all fulfilling the HLH-94/HLH-2004 inclusion criteria. In the current report, patients with GS2 are included in the term FHL. Of these 366 children, 283 had verified molecular diagnoses of FHL and all others (n=83) had verified familial disease (Supplemental Table 1); 72/366 did not fulfil ≥5 diagnostic criteria.

Familial HLH is characterized by systemic inflammation and as controls we used children with other forms of inflammation that could be confused with HLH, more specifically infections and systemic rheumatic diseases[17-19]. Three such cohorts of children with available clinical and laboratory data were utilized: 374 "rheuma-controls" with systemic juvenile idiopathic arthritis (sJIA), all without macrophage activating syndrome (MAS-HLH), and 329 separate children with infections that diagnostically could be confused with having developed MAS-HLH (in children with sJIA) ("inf-controls-1") were both obtained from a study on classification criteria for MAS-HLH complicating sJIA[20,21]. The third cohort (n=361) were children with severe sepsis (defined as presence of suspected infection, ≥2 systemic inflammatory response syndrome (SIRS) criteria, and ≥1 organ failure) at a pediatric intensive care unit (ICU) ("inf-controls-2"); data on 368 children were received, of whom seven were excluded because of known mutations in HLH-related genes (*UNC13D*=5, *PRF1*=1, *XIAP*=1)[22,23]. Of the inf-controls-2, 42 had an underlying malignancy but no other controls. The selection of controls is presented in detail in the Supplemental Text. Since the values from inf-controls-2 were not from the time of diagnosis but instead represent the most abnormal values from a sepsis-related ICU stay, up to 28 days in duration, this cohort was only used for analysis of sCD25 as detailed in "Statistical methods" below. No control patient fulfilled ≥5 diagnostic criteria except seven inf-controls-2, six had six criteria (two with malignancies) and one had seven, but this corresponds to the most abnormal values from their sepsis-related ICU stay. Because of a high degree of missing values in the controls, it is impossible to know who would have fulfilled the diagnostic criteria had they been completely assessed.

Patient characteristics for all cases, all controls, and the respective control cohorts are presented in Table 2. The study was approved by the Ethical Committee in Stockholm (2016/135–31; 2018/1387-32) and the Swedish Ethical Review Authority (2020-02162).

### The clinical diagnostic strategy

The following categorical variables were analyzed statistically: sex, fever, splenomegaly, and hepatomegaly; and the following continuous variables: hemoglobin, absolute neutrophil count, platelet count, triglycerides, fibrinogen, ferritin, sCD25, alanine transaminase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH), bilirubin, albumin, and

creatinine (see Figure 1 for distribution of original data evaluated in cases and the three control groups, and Supplemental Figure 1 for imputed data). Values for hemoglobin, neutrophils, platelets, AST, LDH, and bilirubin in newborn children, aged ≤10 days, were not included in the statistical analyses because they may be misleading. We noticed that inf-controls-1, *i.e.*, children selected so that they could be confused with MAS-HLH, were skewed in the sense that everyone had fever, and therefore the variable fever was not included in the statistical analyses on diagnostic criteria and diagnostic score. Since values for sCD25 were reported in U/mL in cases but in pg/mL in inf-controls-2, and since we could not find an established conversion model from pg/mL to U/mL, we developed such a conversion model (see Supplemental Text and Supplemental Figure 2). To provide complete data sets for the analyses, multiple imputations were performed (see "Approach to missing data" below).

Prior to initiating the statistical analyses, it was decided to statistically evaluate three different methods in the clinical strategy; a) a number of diagnostic criteria fulfilled (*i.e.*, such as "5/8 criteria"), b) a numerical score using cut-offs for continuous laboratory values, and c) a numerical score using a continuous scale for laboratory values. The three methods would be compared both with regard to diagnostic preciseness and how easy they would be to use clinically, and compared with current HLH-2004 criteria. All models are built on training data (70%) and evaluated on test data (30%).

**The diagnostic genetic and cellular pathways**

For these pathways, the criteria are based on the author's expert opinion, in turn based on accumulated experience developed in six countries with long-term specific interest in HLH; Canada, China, Germany, Italy, Sweden, and the United States of America.

**Statistical methods**

The statistical methods for estimation of numerical scores using cut-offs for continuous laboratory values and for estimation of numerical scores using a continuous scale for laboratory values are presented in Supplemental Text.

*Approach to missing data*

To address the problem of missing values, multiple imputation was applied, using 20 imputed datasets for the case and each control group separately. In each of the imputed datasets, the missing values were replaced by imputed values sampled from the distribution of the observed data of all variables[24]. The imputed datasets were then combined for the case and control groups, except for the inf-controls-2 group as these observations were only used to impute values of sCD25 to the inf-controls-1 and rheuma-controls. All analyses conducted were performed on each of the 20 imputed datasets and the results were combined using Rubin's rules[25].

*Number of diagnostic criteria fulfilled*

Of cases and controls, 70% were randomly assigned to a training set and the remaining 30% to a test set. To compare cases and controls, Fisher's exact test for binary variables and Wilcoxon's rank sum test for continuous variables were used. All chosen variables except age were significantly different between the two groups and used in a multivariable logistic regression model, where binary variables were coded as absent or present and continuous variables were dichotomized. The cut-off for each continuous variable was determined based on a receiver operating characteristic (ROC) curve analysis, where sensitivity and specificity were maximized. All calculations above were conducted on each of the 20 imputed datasets and the results were summarised using Rubin's rules.

The binary variables from the multivariable logistic regression model that were significantly associated with the outcome at significance level $p<0.20$, as suggested in[26], were used to calculate a sum of score for each patient in both cases and control groups, such that one fulfilled criterion resulted in one point. This sum was then used in a univariable logistic regression model to calculate the probability of FHL by converting the probability according to $\hat{p}(score) = \frac{e^{\hat{\beta}_0+\hat{\beta}_1*Score,}}{1+e^{\hat{\beta}_0+\hat{\beta}_1*Score,}}$ (for explanations on equation components, see Supplemental Text). The results are presented as probability of FHL for each sum of score with corresponding receiver-operating area under the curve (AUC), accuracy ($\frac{True\ Positive+True\ Negative}{Total\ Sample}$), sensitivity ($\frac{True\ Positive}{True\ Positive+False\ Negative}$), specificity ($\frac{True\ Negative}{True\ Negative+False\ Positive}$), positive predictive value (PPV) ($\frac{True\ Positive}{True\ Positive+False\ Positive}$), and negative predictive value (NPV) ($\frac{True\ Negative}{True\ Negative+False\ Negative}$).

*Assessment of current HLH-2004 criteria*

To test the current HLH-2004 criteria with these control cohorts, the same procedure as described in *Number of diagnostic criteria fulfilled* was applied, with the difference that the cut-off values for the continuous variables were from the current HLH-2004 criteria with some modifications. In these analyses, we used the criterion bicytopenia, as stated in the HLH-2004 criteria, and not individual cytopenias. Since data were missing regarding hemophagocytosis and NK-cell activity for cases and controls, we arbitrarily sampled 50% of the controls to have these variables, respectively, to be compared to 72% and 85%, respectively, of the cases with verified FHL, as in the HLH-2004 study. In addition, accuracy values are presented for alternative frequencies of both of these variables in the controls, ranging from 0% to 100% (Supplemental Table 2). For the percentage of fever in cases and controls, we used the same percentage (92%) as for fever among children with verified FHL in the HLH-2004 study. For an assumption of the prevalence of verified FHL/GS2 in the study population, we used the same percentage as in the Italian HLH Registry as of January 24, 2023, i.e., 16% (99/601); for explanations see *Proportion of cases vs controls* in the Supplemental Text.

## RESULTS

### Three diagnostic strategies

We outline three different strategies for a diagnosis of FHL, based on 1) clinical symptoms and routine laboratory findings, 2) genetic analyses, and 3) cellular analyses of lymphocytes.

### The clinical diagnostic strategy

*Number of diagnostic criteria fulfilled*

Comparing all 366 cases with 703 controls (inf-controls-1 and rheuma-controls) for the 17 variables, except fever and sCD25 for reasons detailed in Methods, seven variables were significant with $p<0.20$ (as suggested in[26]): splenomegaly ($p=0.01$), neutrophils $\leq 2.6x10^9$/L ($p=0.01$), platelets $\leq 136x10^9$/L ($p=0.03$), triglycerides $\geq$2.08mmol/L ($p=0.08$), fibrinogen $\leq$2.6g/L ($p=0.01$), ferritin $\geq$621µg/L ($p=0.06$), and bilirubin $\geq$17µmol/L ($p=0.08$). The number of missing data for each variable and diagnostic group is found in Supplemental Table 3.

Assuming a prevalence of FHL at 16% in our study population (as in the Italian HLH Registry), using these seven significant variables led to an accuracy of 97.7%. Adding fever and sCD25 ($\geq$5,369U/mL) led to an accuracy of 99.1% (Table 3).

To facilitate clinical use, we then evaluated more even cut-off values based on the author's expert opinion but still using the same nine variables; fever, splenomegaly, neutrophils $\leq 1.0x10^9$/L, platelets $\leq 100x10^9$/L, triglycerides $\geq$2.0mmol/L, fibrinogen $\leq$2.5g/L, ferritin $\geq$500µg/L, sCD25 $\geq$5,000U/mL, and bilirubin $\geq$20µmol/L. Here accuracy was 99.1%, AUC 0.9961, sensitivity 97.1%, specificity 99.5%, PPV 97.6%, and NPV 99.4% (Table 3).

*Assessment of the current HLH-2004 diagnostic criteria*

We then analyzed the current HLH-2004 criteria (Table 1), still assuming a 16% prevalence of FHL, which revealed an accuracy of 97.4%. Since cellular assays are here considered a separate diagnostic pathway, we then estimated a model using the current HLH-2004 criteria except NK-cell function, which revealed an accuracy of 99.0%, AUC 0.9924, sensitivity 96.2%, specificity 99.5%, PPV 97.6%, and NPV 99.2% (Table 3).

Since it has been suggested that the cut-offs of ferritin at 500µg/L and sCD25 at 2,400U/mL are too low[27-29], we also evaluated a ferritin cut-off of 1,000µg/L, by which the accuracy increased to 99.4%. By instead increasing the sCD25 cut-off to 5,000U/mL, accuracy decreased to 98.2%, and with ferritin 1,000µg/L and sCD25 5,000U/mL accuracy was 99.1% (Table 3).

Thus, overall the HLH-2004 criteria demonstrated high accuracy, sensitivity and specificity for FHL in this cohort.

*Numerical score using cut-offs for continuous laboratory values*

The seven variables that were significant had an AUC of 0.9980 and at score 100, the probability for FHL was >50%, specifically 59.9%, with an accuracy of 98.1%, sensitivity 98.1%, specificity 98.1%, PPV of 91.1% and NPV of 99.6%.

The total score ranged from 0 to 173. The cases had a mean score of 151 (median 156, interquartile range 140-173). For the controls, the corresponding values were 20, 14, and 4-28. The probability of FHL for various scores are presented in Supplemental Table 4.

*Numerical score using a continuous scale for laboratory values*

In the first logistic regression model, using 14 variables with both binary and continuous outcomes, significant p-values (p<0.20) were obtained for the following variables: splenomegaly (p<0.01), hepatomegaly (p=0.01), hemoglobin (p<0.01), triglycerides (p<0.01), fibrinogen (p<0.01), neutrophils (p<0.01), platelets (p<0.01), ferritin (p=0.07), ALT (p=0.04), LDH (p=0.19), and bilirubin (p=0.05) (Supplemental Table 5).

These significant variables were then included in a final model, in which all above variables were significant at p<0.20 (Supplemental Table 6). This resulted in a model with AUC 0.9976. Inserting the patients values in the equation $\hat{p}(X) = \frac{e^{\hat{\beta}_0+\hat{\beta}X}}{1+e^{\hat{\beta}_0+\hat{\beta}X}}$, provides the probability that the patient has FHL.

**The genetic diagnostic pathway**

Following consensus discussions, we deem that a diagnosis of FHL, potentially necessitating curative treatments, can be based on the presence of biallelic loss-of-function variants, including large deletions, nonsense variants, or previously well described HLH-associated rearrangements, non-coding or missense variants (Table 4). Such variants should be annotated as *pathogenic* in the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/), a freely available archive for interpretations of clinical significance of constitutional and somatic human variants of any size, type or genomic location[30]. The Human Gene Mutation Database (https://my.qiagendigitalinsights.com/bbp/) constitutes a comprehensive collection of published germline mutations in genes that are thought to underlie, or are closely associated with, human inherited disease.

In patients carrying rare coding variants in HLH-associated genes that are novel, deemed *likely pathogenic*, of *unknown significance* or *conflicting interpretation*, assays of lymphocyte cytotoxic function are strongly recommended. In addition, the Genome Aggregation Database (gnomAD, https://gnomad.broadinstitute.org/) is currently the largest

publicly available collection of population variation from harmonized sequencing data[31]. Providing useful insights, the gnomAD browser enables rapid and intuitive variant analysis[32], including prediction of pathogenicity using Combined Annotation Dependent Depletion (CADD) tool scores[33]. In individuals where biallelic possible disease-causing variants are not identified but that suffer repeated episodes of HLH, cellular diagnostic pathways assessing cytotoxic lymphocyte function are strongly recommended to rule out pathogenic non-coding aberrations.

## The cellular diagnostic pathway

Cellular assays of FHL patients originally identified defective lymphocyte cytotoxicity using assays of radioactivity measuring release of $^{51}Cr$ from labelled K562-cells, a cell line devoid of MHC class I expression and universally susceptible to NK-cell-mediated lysis, after incubation with leukocytes isolated from peripheral blood[11]. Due to radioactive safety restrictions and inherent variability, such cytotoxicity assays have largely been replaced with flow cytometric assay quantifying intracellular perforin expression in NK-cells as well as NK-cell exocytic responses (typically measuring induced surface CD107a expression following incubation with target K562-cells)[15,34,35]. Combined exocytosis, i.e., degranulation, assays quantifying both NK-cell and cytotoxic $CD8^+$ T-cell exocytotic responses provide superior sensitivity and specificity[36]. Absent perforin expression or defective exocytosis by patient NK-cells is indicative of familial HLH (Table 4). A suggestion on how the functional cellular assays best should be performed in provided in the Supplemental Text.

## The genetic and cellular pathways also need signs and/or symptoms of HLH

Genetic or cellular data do not diagnose FHL *per se*, in isolation they only indicate a risk for FHL. They should only be considered diagnostic in patients with signs and/or symptoms of HLH. The suggested signs and symptoms are the diagnostic criteria as defined in Table 5 (fever, splenomegaly, cytopenias, hypertriglyceridemia and/or hypofibrinogenemia, hemophagocytosis, and/or elevated ferritin and/or sCD25), as well as isolated CNS inflammation and/or isolated hepatitis/liver failure. Anyone of these could be sufficient diagnostic support in the alternative diagnostic pathways, with sufficient genetic or cellular findings.

# DISCUSSION

The current criteria most widely used for diagnosis of FHL were developed as enrollment criteria for the HLH-2004 treatment protocol 20 years ago and based on expert opinion[9]. Here we have evaluated these clinical criteria relative to other inflammatory disorders. In addition, we present two additional strategies to make the FHL diagnosis in patients with signs/symptoms suggestive of HLH, a genetic pathway and a cellular pathway.

In contrast to the situation when the HLH-2004 diagnostic criteria were developed, the current analyses of clinical criteria could be based on a large cohort of patients with genetically verified FHL/GS2. This is an enormous advantage as compared to many other medical conditions.

When assessing the current HLH-2004 criteria, which was performed late in our analysis, we were surprised by their positive performance, *i.e.*, an accuracy of 97.4%. Possible reasons for this remarkably high performance can be a homogenous group of cases (all have verified FHL) and that the control groups appear to be quite distinct from the cases (Figure 1). However, the possibility of a confirmation bias cannot be excluded, *i.e.*, that the FHL cases were defined on fulfilling HLH-2004 criteria which thus formed the basis for genetic analyses.

Using the current HLH-2004 criteria except NK-cell cytotoxicity the accuracy was 99.0%. By modifying the ferritin level from 500 to 1,000 μg/L or the sCD25 level from 2,400 to 5,000 U/mL, the accuracy was 99.4% and 98.2%, respectively (Table 3). However, the authors deem that there is no meaningful difference between the various alternatives, and therefore not sufficient reasons to change the cut-off levels of ferritin and sCD25, also having in mind the value of easy comparison with studies over the last 20 years that used the current cut-off levels. Moreover, by using lower cut-off levels, more patients will fulfil the criteria and, with reduced costs of genetic sequencing, it is valuable that more patients are sequenced. The suggested revised clinical criteria for FHL are presented in Table 5.

When the current study started, we considered three different methods for the clinical pathway; a number of diagnostic criteria fulfilled, a numerical score using cut-offs for continuous laboratory values resembling the HScore[37], and a numerical score using a continuous scale for laboratory values. In our corresponding 'FHL-score', at the best cut-off of 100 points, accuracy was 98.1%; *i.e.*, lower than the accuracy of 99.0% reached at five of seven fulfilled HLH-2004 criteria, *i.e.*, except NK-cell activity, in the current study (Table 3). We, therefore, do not suggest this somewhat more complicated model, nor a model with a numerical score using a continuous scale for laboratory values since this would need a computer or a website to estimate the probability for FHL.

The cellular diagnostic pathway appears to provide quite reliable prediction on the likelihood that the patient has FHL and, moreover, results can be provided already within a day in some laboratories (Table 4). The sensitivity of cytotoxic lymphocyte exocytosis assays can be further augmented using different triggers of NK-cell activation or also examining cytotoxic $CD8^+$ T-cell responses[38]. In patients with recurrent HLH but in which genetic analysis fail to provide a molecular diagnosis, cytotoxicity assays should be repeated when used as a basis for instigating curative treatments associated with significant side-effects. In patients with novel genetic associations, further cellular assays can be useful to determine pathophysiological processes[39]. Notably, to avoid potential pitfalls, cytotoxicity assays should be performed by experienced laboratories. A drawback is that there are few well experienced

laboratories, and another drawback is that reliable results require fresh cells, *i.e.*, ideally the sample is delivered to the laboratory within around 24 hours after sampling.

Notably, aberrations in a large number of other genes than FHL2-5 and *RAB27A* have been associated with HLH, including mutations in *LYST* (Chediak-Higashi Syndrome), *SAP* (XLP-1), *XIAP* (XLP-2), and *NLRC4*[40]. HLH can also be a less common manifestation of other genetic diseases including several immunodeficiencies, autoinflammatory diseases and lysinuric protein intolerance. However, high-throughput sequencing technologies are now established in clinical laboratories for the rapid genome-wide investigation and diagnosis of patients suffering life-threatening constitutional genetic diseases.

Hypomorphic variants in HLH-associated genes and multiple variants in distinct genes represent a challenge to interpretation of a molecular diagnosis, supporting the value of the cellular pathway. An example of a hypomorphic variant frequently encountered in late-onset cases of HLH is *PRF1* c.272C>T (p.Ala91Val), which in a biallelic setting confers an approximate 50% reduction in perforin expression and lymphocyte cytotoxic function yet has an allelic frequency of 5% in some populations[41]. Furthermore, experimental evidence from animal models of HLH indicate that an increasing polygenic burden of loss-of-function variants in HLH-associated genes correlates with augmented predisposition to HLH[42]. Clinical evidence supports a potential di- or polygenic mode of inheritance of familial HLH in which single loss-of-function mutations in two different degranulation pathway genes cooperate to impair cellular cytotoxicity[43].

When to use each diagnostic pathway depend on the clinical situation. The clinical pathway can rapidly help to decide on whether to initiate HLH-directed therapy. The cellular pathway may at a later stage give additional prediction on the likelihood of FHL. Importantly, although the clinical and cellular pathways both independently may be strongly indicative of FHL, and be the basis for initiating pre-HSCT treatments, we deem that the FHL diagnosis should be confirmed through genetic analysis, such as for decisions on performing HSCT. Moreover, the genetic and cellular pathways can both diagnose patients before disease presentation, enabling rapid HSCT that reduces risk of sequelae. As a diagnostic complement, the MAS/HLH (MH) score can be used to discriminate between primary HLH and MAS-HLH[44].

Our study has several weaknesses, one is that our analyses are based on the control groups used and, strictly speaking, our data are relevant only for the control groups used. Thus, since malignancies and atypical infections were not included as controls, such diseases have to be excluded in each patient. Another weakness is that there were several missing values in the data sets, and as a result, multiple imputations were applied. The extent of imputation could overestimate the PPV and NPV of the clinical FHL criteria, and some variables deemed not to be statistically different between controls and FHL may have been underestimated, but multiple imputations are still considered reasonable with limited bias[45]. Since there were no controls with known secondary HLH, and since the syndrome HLH may have various possible causes[46], it is always of vital importance to search for the underlying reason(s) for the HLH, either genetic, as in FHL, or acquired, such as infections, malignancies or

inflammatory disorders, as in secondary HLH. This includes sepsis-associated HLH, so that appropriate therapy is provided. Notably, the pattern of inflammatory cytokines can help to diagnose HLH early, to discriminate HLH from non-HLH as well as the type of HLH; an approach that is encouraged[47-51]. Moreover, as always in medicine, making a clinical diagnosis should be based on the entire evaluation of the patient, and cannot be entirely based on pre-defined clinical criteria.

To conclude, we present three separate pathways to the diagnosis of FHL, a clinical, a cellular based on cytotoxicity, and a genetic pathway. As clinical criteria, we suggest to keep the HLH-2004-based criteria, except NK-cell activity. We hope that these three pathways will be helpful in making the diagnosis of FHL in the future.

## ACKNOWLEDGEMENTS

The work was supported by grants from the Swedish Childhood Cancer Fund (KP2021-0006), the Swedish Cancer Society (Dnr: 19 0362 Pj), the Cancer and Allergy Foundation of Sweden (Ref no: 10118), and Region Stockholm (ALF-project) (FoUI-960717). We thank Dr Maurizio Aricò, Italy, for his valuable contributions to management of FHL and for his involvement in the early phase of this study.

## AUTHORSHIP CONTRIBUTION

JIH, ACH, and MBJ planned the study, with JIH as principal investigator. JIH, EB, ACH, MLC, and ES provided data summaries on cases. FM, KFK, RQC, AR, and SWC, provided data summaries on controls. JE, IHM and MB performed statistical analyses. YTB and ES developed the format for the genetic and functional pathways. JIH, ACH, JE, ARK, KL, AN, YMT, MBJ, ES, and YTB analyzed data. JIH drafted the manuscript, assisted by JE and YTB, and it was reviewed and approved by all authors.

## CONFLICT OF INTEREST DISCLOSURES

JIH, ES, RC, ARK, KL, ACH, and MBJ serve as consultants and/or in advisory boards for SOBI. MBJ also has research support from SOBI. RC also serves as consultant for Pfizer, Abbvie, Curbside Consults, Vindico Medical Education, Lilly, and unpaid consultant for APOLLO Therapeutics. ACH also serves as a speaker for Novartis. KK's work is funded by K23GM148827. AN is on the advisory board for JAZZ pharmaceuticals. SC has received consulting fees from Apollo and Simcha therapeutics, and speaker fees from SOBI and PracticePoint communications, JE, EB, IHM, MLC, FM, AR, YMT, MB and YTB have no conflicts to declare.

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## Figure Legend

**Figure 1. Beeswarm panels of all imputed continuous variables evaluated in cases (n=366) and the three control groups**: hemoglobin, absolute neutrophil count, platelet count, triglycerides, fibrinogen, ferritin, soluble CD25, alanine transaminase, aspartate transaminase, lactate dehydrogenase, bilirubin, albumin, and creatinine. These analyzed values presented include imputated values. 'Infection-1' includes 329 children with systemic infections that could be confused with MAS-HLH. 'Infection-2' includes 361 children with severe sepsis treated at a pediatric intensive care unit. 'Rheuma' includes 374 children with systemic juvenile idiopathic arthritis, all without MAS-HLH.

**Table 1: Diagnostic criteria for the HLH-2004 trial, evaluated in the current study.**

The diagnosis HLH can be established if one of either 1 or 2 below is fulfilled:

***1. A molecular diagnosis consistent with HLH***

***2. Diagnostic criteria for HLH fulfilled (5 out of the 8 criteria below)***

* Fever
* Splenomegaly
* Cytopenias (affecting ≥ 2 of 3 lineages in the peripheral blood:
  Hemoglobin (<90 g/L); Platelets (<100 x $10^9$/L); Neutrophils (<1.0 x $10^9$/L)
  (In infants <4 weeks: Hemoglobin <100 g/L)
* Hypertriglyceridemia and/or hypofibrinogenemia
  Fasting triglycerides ≥3.0 mmol/L; Fibrinogen ≤1.5 g/L
* Hemophagocytosis
* Low or absent NK-cell activity (according to local laboratory reference)
* Ferritin ≥500 µg/L
* Soluble CD25 (*i.e.*, soluble interleukin-2 receptor) ≥2,400 U/mL

**Table 2: Patient characteristics for all patients, all controls, and the respective controls cohorts.**

| | Children with FHL | Controls (n=1,064) | Inf-controls-1 (n=329) | Inf-controls-2 (ICU) (n=361) | Rheuma-controls (n=374) |
|---|---|---|---|---|---|

| | **(n=366)** | | | | |
|---|---|---|---|---|---|
| | n yes / n evaluated / % yes of total | | | | |
| Male sex | 189 / 366 / 51.6 % | 554 / 1,064 / 52.1 % | 166 / 329 / 50.5 % | 204 / 361 / 56.5 % | 184 / 374 / 49.2 % |
| Fever | 331 / 363 / 90.4 % | 836 / 1,063 / 78.6 % | 329 / 329 / 100 % | 152 / 360 / 42.1 % | 355 / 374 / 94.9 % |
| Splenomegaly | 349 / 366 / 95.4 % | 131 / 1,059 / 12.3 % | 20 / 328 / 6.1 % | 25 / 361 / 6.9 % | 86 / 370 / 23 % |
| Hepatomegaly | 308 / 342 / 84.2 % | 225 / 1,060 / 21.1 % | 32 / 328 / 9.7 % | 77 / 361 / 21.3 % | 116 / 371 / 31 % |
| | median (mean; IQR) / n evaluated | | | | |
| Age at start of HLH–2004 (days) | 0.294 (1.88; 0.162–1.37) / 366 | 4.48 (5.70; 1.68-9.16) / 1,604 | 3.33 (4.92; 1.45–7.92) / 329 | 4.85 (5.92; 1.09-10.4) / 361 | 5.49 (6.18; 2.58–9.56) / 374 |
| Hemoglobin (g/L) | 75.0 (75.8; 65.0–84.8) / 342 | 105 (105; 92.0–118) / 1,044 | 118 (116; 108–127) / 326 | 96.0 (97.9; 86.0–108) / 355 | 101 (102; 90.0–113) / 363 |
| Neutrophils (ANC) (x $10^9$/L) | 0.60 (1.25; 0.295–1.20) / 311 | 8.62 (10.2; 4.06-14.0) / 910 | 6.84 (8.93; 3.73–11.8) / 323 | 6.84 (8.97; 2.69-12.8) / 330 | 11.6 (13.3; 7.65–17.4) / 257 |
| Platelets (x $10^9$/L) | 31.0 (48.6; 17.8–56.0) / 340 | 325 (348; 194–480) / 1,042 | 342 (361; 260–443) / 327 | 149 (172; 72.3-235) / 354 | 498 (510; 375–614) / 361 |
| Triglycerides (mmol/L) | 3.67 (4.18; 2.49–5.46) / 338 | 1.40 (1.79; 1.09-1.86) / 366 | 1.50 (1.75; 1.21–1.84) / 122 | 1.52 (2.22; 1.01-2.99) / 116 | 1.36 (1.45; 1.00–1.65) / 128 |
| Fibrinogen (g/L) | 1.00 (1.39; 0.690–1.80) / 324 | 5.13 (5.32; 3.81–6.59) / 330 | 4.01 (4.43; 2.90–5.60) / 132 | | 5.60 (5.91; 4.63–7.20) / 198 |
| Ferritin (µg/L) | 2,590 (7,940; 1,200–7,640) / 327 | 188 (1340; 76.0–624) / 810 | 67.0 (156; 32.0–126) / 201 | 212 (1790; 95.4-605) / 361 | 462 (1,660; 157–1,600) / 248 |
| Soluble CD25 (U/mL) | 20,700 (23,000; 9,750–29,800) / 113 | 2,050 (2,810; 1,370–3,080) / 361 | | 2,050 (2,810; 1,370-3,080) / 361 | |
| Aspartate transaminase (U/L) | 162 (317; 75.0–352) / 288 | 33.0 (49.7; 24.0–44.0) / 661 | 34.0 (61.7; 25.0–46.0) / 311 | | 32.0 (39.0; 22.0–42.0) / 350 |
| Alanine transaminase (U/L) | 125 (205; 56.3–266) / 320 | 27.0 (87.1; 15.0–41.0) / 938 | 22.0 (48.8; 14.0–36.0) / 314 | 35.0 (190; 21.0-85.0) / 297 | 25.0 (30.5; 13.0–35.0) / 327 |
| Lactate dehydrogenase (U/L) | 704 (1,070; 493–1,170) / 286 | 442 (517; 314–608) / 488 | 470 (529; 338–615) / 236 | | 427 (507; 302–598) / 252 |
| Bilirubin (µmol/L) | 23.1 (58.2; 13.3–78.1) / 281 | 8.55 (17.8; 5.13–15.4) / 722 | 7.52 (12.8; 4.28–13.7) / 215 | 10.3 (27.8; 5.13–22.2) / 288 | 6.84 (9.55; 5.00–11.3) / 219 |
| Creatinine (µmol/L) | 27.0 (37.6; 19.1–44.2) / 266 | 36.3 (50.1; 26.5–54.8) / 999 | 35.4 (41.5; 25.7–52.2) / 317 | 35.4 (59.0; 22.1-55.7) / 361 | 44.2 (48.5; 32.7–61.9) / 321 |
| Albumin (g/L) | 28.0 (28.2; 24.0–32.0) / 265 | 38.0 (37.4; 33.0–42.0) / 521 | 39.6 (39.1; 35.4–43.0) / 264 | | 35.6 (35.7; 30.8–40.0) / 257 |

**Table 3: Summary of statistical results for various options of the clinical diagnostic pathway and various combinations of variables.***

| Option of clinical pathway | Variables included | No. of criteria** | AUC*** | Accu-racy | Sensiti-vity | Specifi-city | PPV | NPV |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |
| ***Number of diagnostic criteria fulfilled*** | Exact values based on current optimization[#] | 4/7 | 0.9976 | 97.7% | 98.1% | 97.6% | 89.1% | 99.6% |
| | - as above + fever + sCD25[##] | 6/9 | 0.9991 | 99.1% | 97.1% | 99.5% | 97.6% | 99.4% |
| | Rounded values (ferritin ≥500 µg/L)[§] | 5/9 | 0.9961 | 99.1% | 97.1% | 99.5% | 97.6% | 99.4% |
| | Rounded values (ferritin ≥1,000 µg/L)[§] | 5/9 | 0.9967 | 99.1% | 97.1% | 99.5% | 97.6% | 99.4% |
| | | | | | | | | |
| HLH-2004 criteria | Current criteria¤ | 5/8 | 0.9920 | 97.4% | 99.0% | 97.1% | 87.3% | 99.8% |
| | Current criteria except natural killer cell activity¤ | 5/7 | 0.9924 | 99.0% | 96.2% | 99.5% | 97.6% | 99.2% |
| | - ferritin ≥500 µg/L, sCD25 ≥2,400 U/mL | 4/7 | 0.9924 | 93.8% | 99.0% | 92.8% | 73.3% | 99.8% |
| | - ferritin ≥1,000 µg/L, sCD25 ≥2,400 U/mL | 5/7 | 0.9932 | 99.4% | 96.2% | 100% | 100% | 99.2% |
| | - ferritin ≥1,000 µg/L, sCD25 ≥2,400 U/mL | 4/7 | 0.9932 | 94.6% | 99.0% | 93.8% | 76.0% | 99.8% |
| | - ferritin ≥500 µg/L, sCD25 ≥5,000 U/mL | 5/7 | 0.9951 | 96.2% | 96.2% | 100% | 100% | 99.2% |
| | - ferritin ≥500 µg/L, sCD25 ≥5,000 U/mL | 4/7 | 0.9951 | 98.2% | 99.0% | 98.1% | 91.2% | 99.8% |
| | - ferritin ≥1,000 µg/L, sCD25 ≥5,000 U/mL | 5/7 | 0.9954 | 99.4% | 96.2% | 100% | 100% | 99.2% |
| | - ferritin ≥1,000 µg/L, sCD25 ≥5,000 U/mL | 4/7 | 0.9954 | 99.1% | 99.0% | 99.0% | 95.4% | 99.8% |
| | | | | | | | | |
| ***Numerical score using cut-offs for continuous laboratory values*** | At score 100, FHL probability was 59.9%. Variables: Splenomegaly, neutrophils, platelets, triglycerides, fibrinogen, ferritin, bilirubin | | 0.9980 | 98.1% | 98.1% | 98.1% | 91.1% | 99.6% |
| | | | | | | | | |
| ***Numerical score using a continuous scale for laboratory values*** | Variables: splenomegaly, hepatomegaly, hemoglobin, neutrophils, platelets, triglycerides, fibrinogen, ferritin, ALT, LDH, bilirubin | | 0.9976 | NA | NA | NA | NA | NA |

Abbrev: ALT, alanine transaminase, AUC, area under the curve; LDH, lactate dehydrogenase; NA, not applicable; NPV, negative predictive value; PPV, positive predictive value.

* Assumption: A prevalence of FHL of 16% in the study population, *i.e.*, similar as in the Italian HLH Registry.

** The number of criteria presented here are the numbers that maximizes the sensitivity and specificity.

*** AUC is calculated based on all criteria and not just five out of seven criteria, or similar.

# Splenomegaly, neutrophils ≤2.7x$10^9$/L, platelets ≤136x$10^9$/L, triglycerides ≥2.08 mmol/L, fibrinogen ≤2.6 g/L, ferritin ≥621 µg/L, and bilirubin ≥17 µmol/L.

## As above, as well as fever and sCD25 ≥5,369 U/mL.

§ Fever, splenomegaly, neutrophils ≤1.0x$10^9$/L, platelets ≤100x$10^9$/L, triglycerides ≥2.0 mmol/L, fibrinogen ≤2.5 g/L, ferritin ≥500 µg/L or 1,000 µg/L, sCD25 ≥5,000 U/mL, and bilirubin ≥20 µmol/L.

¤ Ferritin ≥500 µg/L and sCD25 ≥5,000 U/mL.

**Table 4: Alternative pathways to diagnose FHL: the genetic and the cellular pathways**

| **THE GENETIC PATHWAY: Genetic observation** | **Predictive value** | **Recommended diagnostic follow-up** |
|---|---|---|
| Biallelic combination of large deletions, nonsense variants, and other previously well described disease-causing* genetic aberrations in FHL-associated genes | Sufficient for FHL diagnosis | Not necessary, but functional validation can be supportive |
| Biallelic combination of large deletions, nonsense mutations, or previously described genetic aberrations with at least one rare missense or non-coding genetic aberration in an FHL-associated gene | Suspicion of FHL | Other diagnostic validation necessary, functional validation recommended |
| Genetic variant of unknown significance identified in an FHL-associated gene | Undetermined | Other diagnostic validation necessary, functional validation recommended |
| No genetic variants identified in FHL-associated genes | Undetermined | Other diagnostic validation necessary, functional validation can be supportive |
| | | |
| **THE CELLULAR PATHWAY: Functional cellular observation** | | |
| Absent perforin expression or defective cytotoxic lymphocyte exocytosis[#] | Strong suspicion of FHL | Genetic analysis necessary |
| Low perforin expression or impaired cytotoxic lymphocyte exocytosis[#] | Suspicion of FHL | Genetic analysis necessary. Repeated functional analyses recommended. |
| Normal perforin expression and cytotoxic lymphocyte exocytosis[#] | FHL less likely | Genetic analyses suggested if FHL is clinically suspected |

* With documented cases of Mendelian inheritance

# The definitions of defective, low, impaired and normal are specific for each laboratory and cannot be generalized

**Table 5: Revised diagnostic criteria for the diagnosis of FHL**

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The diagnosis of FHL can be established if at least one of either 1, 2 or 3 below is fulfilled*:

***1. A molecular diagnosis consistent with FHL in a patient with signs / symptoms suggestive of HLH***

***2. Functional cellular findings consistent with FHL in a patient with signs / symptoms suggestive of HLH***

***3. Clinical diagnostic criteria for FHL with at least 5 out of the 7 criteria below fulfilled***

* Fever ≥38.5$^{o}$C
* Splenomegaly (≥2 cm below the costal margin)
* Cytopenias (affecting ≥ 2 of 3 lineages in the peripheral blood:
  Hemoglobin (<90 g/L); Platelets (<100 x $10^{9}$/L); Neutrophils (<1.0 x $10^{9}$/L)
  (In infants <4 weeks: Hemoglobin <100 g/L)
* Hypertriglyceridemia and/or hypofibrinogenemia
  Fasting triglycerides ≥3.0 mmol/L; Fibrinogen ≤1.5 g/L
* Hemophagocytosis
* Ferritin ≥500 µg/L
* Soluble CD25 (*i.e.*, soluble interleukin-2 receptor) ≥2,400 U/mL

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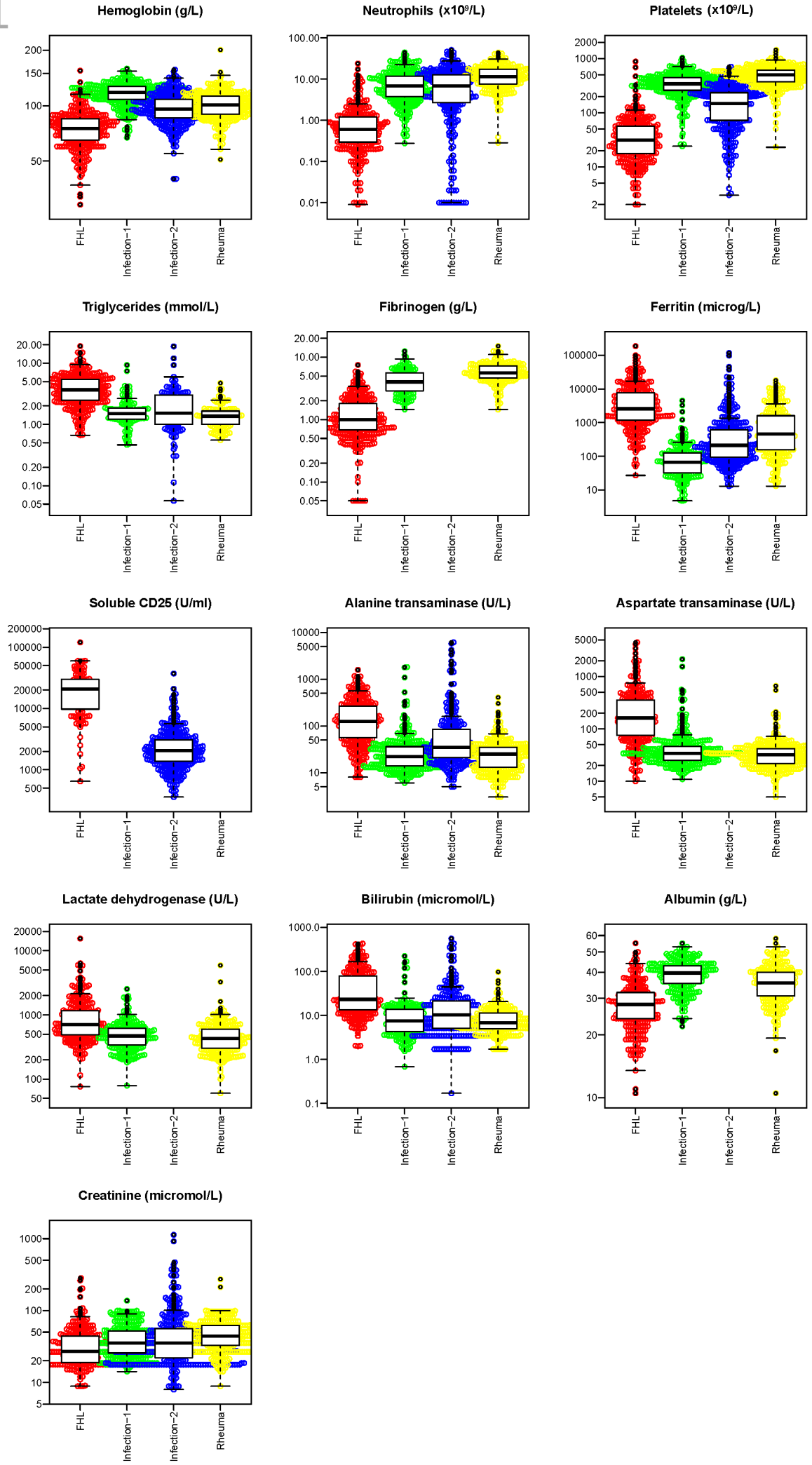
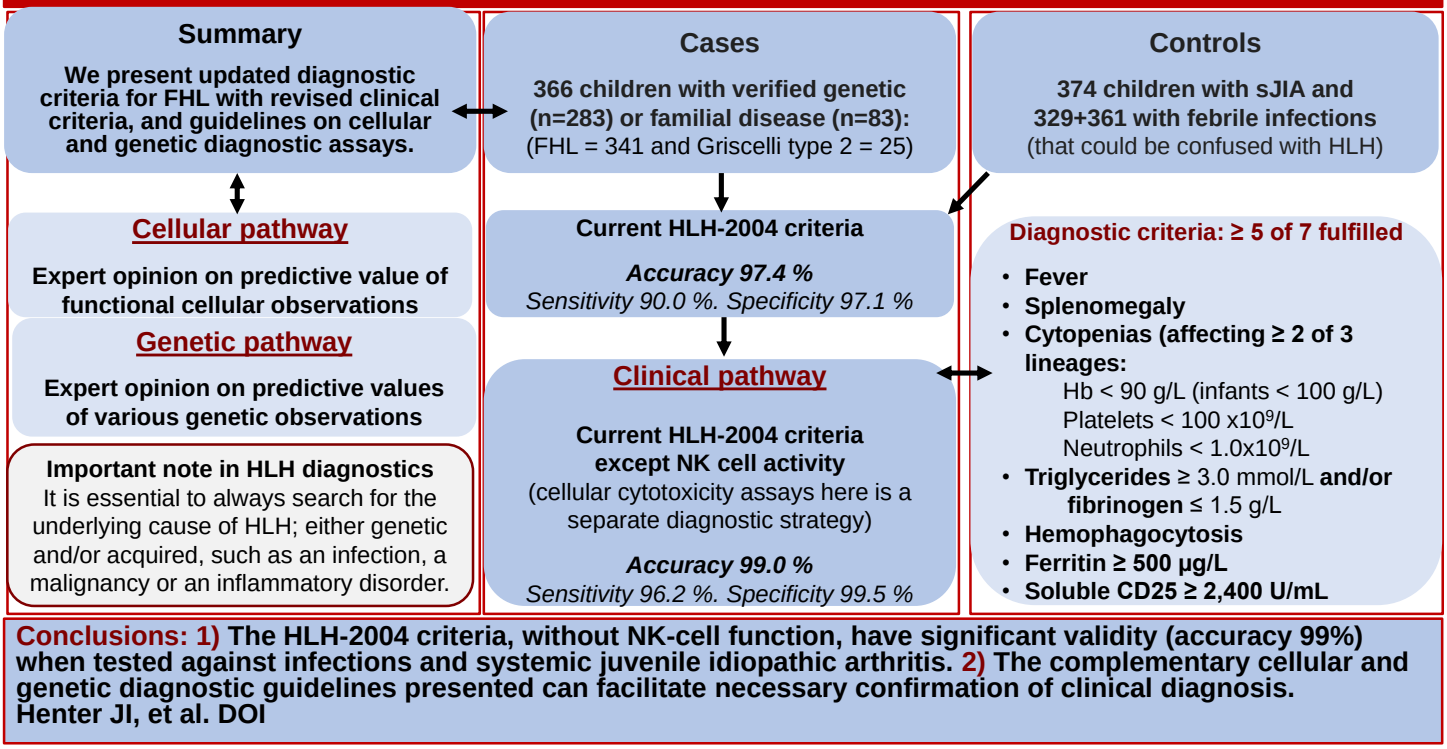
* It is always of vital importance to search for the underlying cause(s) for the HLH syndrome, which may be either genetic, such as FHL, and/or acquired, such as an infection, a malignancy or an autoimmune or autoinflammatory disorder.

Figure 1



# Diagnostic Guidelines for Familial Hemophagocytic Lymphohistiocytosis Revisited




**Conclusions: 1) The HLH-2004 criteria, without NK-cell function, have significant validity (accuracy 99%) when tested against infections and systemic juvenile idiopathic arthritis. 2) The complementary cellular and genetic diagnostic guidelines presented can facilitate necessary confirmation of clinical diagnosis.**
**Henter JI, et al. DOI**