Published in final edited form as:

J Med Genet. 2011 May ; 48(5): 343–352. doi:10.1136/jmg.2010.085456.

Genotype–phenotype study of familial haemophagocytic lymphohistiocytosis type 3

Elena Sieni¹, Valentina Cetica¹, Alessandra Santoro², Karin Beutel^{1,3}, Elena Mastrodicasa⁴, Marie Meeths^{5,6}, Benedetta Ciambotti¹, Francesca Brugnolo¹, Udo zur Stadt⁷, Daniela Pende⁸, Lorenzo Moretta⁹, Gillian M Griffiths¹⁰, Jan-Inge Henter⁵, Gritta Janka³, and Maurizio Aricò¹

¹Department of Pediatric Hematology and Oncology, Azienda Ospedaliero-Universitaria Meyer, Florence, Italy

²U.O. Ematologia I, A.O.Ospedali Riuniti Villa Sofia-Cervello, Palermo, Italy

³Oncology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

⁴S.C. di Oncoematologia Pediatrica con Trapianto di CSE, Ospedale “S.M. della Misericordia” A.O. Perugia, Italy

⁵Childhood Cancer Research Unit, Department of Women’s and Children’s Health, Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden

⁶Clinical Genetics Unit, Department of Molecular Medicine and Surgery, Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden

⁷Research Institute Children’s Cancer Center, Hamburg, Germany

⁸Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy

⁹IRCCS Istituto Giannina Gaslini, Genoa, Italy

¹⁰Cambridge Institute for Medical Research, Addenbrooke’s Hospital, Cambridge, UK

Abstract

Background—Mutations of *UNC13D* are causative for familial haemophagocytic lymphohistiocytosis type 3 (FHL3; OMIM 608898).

Objective—To carry out a genotype–phenotype study of patients with FHL3.

Methods—A consortium of three countries pooled data on presenting features and mutations from individual patients with biallelic *UNC13D* mutations in a common database.

Results—84 patients with FHL3 (median age 4.1 months) were reported from Florence, Italy (n=54), Hamburg, Germany (n=18), Stockholm, Sweden (n=12). Their ethnic origin was

Correspondence to Dr Maurizio Aricò, Dipartimento Oncoematologia Pediatrica, Azienda Ospedaliero-Universitaria Meyer, Viale Pieraccini, 24, 50139 Firenze; m.arico@meyer.it.

Ethics approval This study was conducted with the approval of the Department of Pediatric Hematology and Oncology, Azienda Ospedaliero-Universitaria Meyer, Florence, Italy.

Provenance and peer review Not commissioned; externally peer reviewed.

Caucasian (n=57), Turkish (n=10), Asian (n=7), Hispanic (n=4), African (n=3) (not reported (n=3)). Thrombocytopenia was present in 94%, splenomegaly in 96%, fever in 89%. The central nervous system (CNS) was involved in 49/81 (60%) patients versus 36% in patients with FHL2 (p=0.001). A combination of fever, splenomegaly, thrombocytopenia and hyperferritinaemia was present in 71%. CD107a expression, NK activity and Munc 13-4 protein expression were absent or reduced in all but one of the evaluated patients. 54 different mutations were observed, including 15 new ones: 19 missense, 14 deletions or insertions, 12 nonsense, nine splice errors. None was specific for ethnic groups. Patients with two disruptive mutations were younger than patients with two missense mutations (p<0.001), but older than comparable patients with FHL2 (p=0.001).

Conclusion—*UNC13D* mutations are scattered over the gene. Ethnic-specific mutations were not identified. CNS involvement is more common than in FHL2; in patients with FHL3 and disruptive mutations, age at diagnosis is significantly higher than in FHL2. The combination of fever, splenomegaly, thrombocytopenia and hyperferritinaemia appears to be the most easily and frequently recognised clinical pattern and their association with defective granule release assay may herald FHL3.

INTRODUCTION

Haemophagocytic lymphohistiocytosis (HLH) is a genetically heterogeneous disorder characterised by a hyperinflammatory syndrome with fever, hepatosplenomegaly, cytopenia, and frequently also central nervous system (CNS) involvement.¹ Bone marrow aspiration shows haemophagocytosis by activated macrophages. In most cases the natural course of HLH is rapidly fatal within a few weeks, unless appropriate immune suppressive and cytoreductive treatment by agents including corticosteroids, ciclosporin, etoposide, anti-thymocyte globulin, can obtain transient disease control.²³ So far, haematopoietic stem cell transplantation appears to be the only curative treatment.^{4–8}

Differential diagnosis of HLH may be difficult.⁹ For this purpose, diagnostic guidelines for HLH have been established by the Histiocyte Society.^{10,11} In particular, demonstration of frequent association with common pathogens, together with evidence of impaired natural killer cytotoxic activity, provided the rationale for considering HLH as a selective immune deficiency.^{12–14} Starting from the original report by Farquhar and Claireaux in 1952,¹⁵ autosomal recessive inheritance was proposed and then confirmed as the common mode of inheritance for the familial form of HLH (FHL).

Linkage analysis identified a candidate genomic region on chromosome 9q21.3–22 (FHL1, OMIM 603552).¹⁶ However, the causative gene responsible for the disease has not yet been identified. A simultaneous report established a linkage with another region, 10q21–22,¹⁷ in which the perforin (*PRF1*) gene was identified as responsible for a relevant proportion of cases of FHL (FHL2, OMIM 603553).¹⁸ In patients with FHL2, *PRF1* mutations reduce or abolish the synthesis of the perforin protein, resulting in an impairment of the granule-mediated cytotoxic machinery of NK and T cells.^{19–22} In 2003 a third locus, 17q25, was reported in linkage with FHL (FHL3, OMIM 608898).²³ The involved gene *UNC13D* encodes a protein named Munc13-4, which is thought to contribute to the priming of the secretory granules before they fuse into the plasma cell membrane. Mutations in this gene

impair the delivery of the effector proteins perforin and granzymes into the target cells, resulting in defective cellular cytotoxicity and a clinical picture that appears identical to that associated with *PRF1* mutations. On the basis of a genome-wide screening in a highly consanguineous Kurdish family with FHL, a fourth chromosomal region (6q24) has been reported (FHL4, OMIM 603552).²⁴ Mutations of the syntaxin 11 gene, mapped in this region, are thought to alter intracellular vesicle trafficking of the phagocytic system.²⁵ Recently, zur Stadt *et al* allocated a novel FHL type, FHL5 (OMIM 613101), to a 1 Mb region on chromosome 19p using high-resolution single nucleotide polymorphism genotyping in eight unrelated patients with FHL from consanguineous families. They identified mutations in *STXBP2*, encoding syntaxin binding protein 2 (Munc18-2), a protein involved in the regulation of vesicle transport to the plasma membrane. The 12 patients with FHL5 originated from Turkey, Saudi Arabia and Central Europe.²⁶ Almost simultaneously, a similar report was provided by Côte *et al*.²⁷

The exact contribution of the various mutations in FHL-related genes can only be evaluated in large cohorts. A genotype–phenotype study of FHL2 due to *PRF1* mutations, was reported a few years ago by an international consortium for the Histiocyte Society.²⁸ Nothing comparable has been performed so far for FHL3, the other large subgroup of this disease. For this purpose, we pooled data from three European referral centres, allowing collection of the largest series of patients with FHL3.

METHODS

Data collection

The consortium established between Italy, Germany and Sweden pooled in a common database data on ethnicity, family history, presenting features, mutations and cytotoxic function from individual patients with FHL3 diagnosed on the basis of documented biallelic *UNC13D* mutations. HLH was defined by the diagnostic criteria established by the Histiocyte Society.¹⁰¹¹ CNS involvement was defined as the presence of at least one of the following items: neurological symptoms, cerebrospinal fluid (CSF) pleocytosis (> 5 cells/mm³), elevated CSF protein (> 30 mg/dl), MRI alteration. All data were stored in a common database and analysed. The completeness of the data was as follows: ethnicity, 95%; consanguinity, 95%; age at diagnosis, 98%; persistent fever, 96%; splenomegaly, 96%; CNS disease, 95%; haemoglobin 85%; neutrophils, 89%; platelets, 92%; triglycerides, 75%; fibrinogen, 70%; ferritin, 67%; haemophagocytosis, 96%; CD107 expression, 36%; Munc protein expression, 16%; NK activity, 53%.

Informed consent for the genetic study and the data collection was obtained from the parents or legal guardian at the participating centres. The study was approved by the local international review board at all the participating centres.

UNC13D gene analysis

Genomic DNA was isolated from peripheral blood samples using BioRobot EZ1 Workstation (Qiagen, Jesi, Italy). Some samples were retrieved from our DNA library of patients previously diagnosed.¹ 1928–30 The 32 coding exons and exon–intron boundaries of

the *UNC13D* gene were amplified and directly sequenced, in both directions, with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California, USA). Amplification reactions were performed with 60 ng of DNA, 10 ng of each primer, 200 μ M dNTPs, 1 $\times$ PCR reaction buffer and 2.5 U Taq polymerase in a final volume of 25 μ l; primers are available upon request. Sequences obtained using an ABI Prism 3130XL Sequence Detection System (Applied Biosystems) were analysed and compared with the reported gene structure (gene number 201294, NCBI) using the dedicated software SeqScape (Applied Biosystems). Mutations were confirmed in the parents.

In silico analysis

All mutations were searched in dbSNP (<http://www.ncbi.nlm.nih.gov/snp/>). Unknown mutations were tested by bioinformatic facilities in order to predict whether an amino acid substitution might have functional effect. We used two web query tools: SIFT (Sorting Intolerant From Tolerant: <http://blocks.fhcrc.org/sift/SIFT.html>), which is based on sequence homology, defines tolerated or not tolerated protein changes with a score ranging from 0 to 1 (the amino acid substitution is predicted to be damaging if the score is ≤ 0.05 , and tolerated if the score is >0.05); and POLYPHEN (prediction of functional effect of human nsSNPs: <http://genetics.bwh.harvard.edu/pph/>), which considers phylogenetic and structural information and defines a substitution as 'probably damaging' with a score >2 , 'possibly damaging' 1.5–2 and 'benign' <1.5 . Cryptic splice sites were predicted by NNSplice software (<http://www.fruitfly.org/sequence/human-datasets.html>).

Functional analyses

Peripheral blood mononuclear cells (PBMCs) from patients with FHL3 and healthy donors were isolated by Ficoll gradient centrifugation. NK cells were also purified using the RosetteSep method (StemCell Technologies, Vancouver, British Columbia, Canada), according to the manufacturer's instructions. NK cells were cultured on irradiated feeder cells in the presence of 2 μ g/ml phytohaemagglutinin (Sigma-Aldrich, Irvine, UK) and 100 U/ml recombinant interleukin 2 (Proleukin, Chiron Corp, Emeryville, USA) to obtain high numbers of polyclonal activated NK cell populations. To analyse the cytolytic activity in 4 h ^{51}Cr -release assays, PBMCs were tested against K562, while activated NK cells were tested against the HLA-class I⁺ B-EBV cell line 721.221, demonstrated to be suitable effector/target combinations to reveal cytolytic defect of patients with FHL3.³⁰ E:T ratios ranging from 100:1 to 1:1 were used for PBMCs as effector cells, and from 8:1 to 0.5:1 for activated NK. Lytic units (LU) at 30% lysis were calculated.

PBMCs and activated NK cells were also tested in a degranulation assay quantifying cell surface CD107a expression upon co-culture with K562, as previously described.³⁰ All reagents were from BD Biosciences (Oxford, UK). Briefly, anti-CD107a-PE monoclonal antibody was added before incubation for 3 h at 37°C in 5% CO₂. Thereafter, the cells were stained with anti-CD56-APC and anti-CD3-PerCP monoclonal antibody and analysed by flow cytometry (FACSCalibur, Becton Dickinson, Buccinasco Milano, Italy). Surface expression of CD107a was assessed in the CD3⁺ CD56⁺ cells. Results are reported as CD107a (ie, percentage CD107a⁺ cells of stimulated – percentage CD107a⁺ cells of unstimulated sample).

Western blot analysis

Western blot analysis of Munc13-4 protein was performed as previously described.²⁹

Genotype–phenotype correlations

To explore the correlations between different mutations and clinical, laboratory and functional parameters, *UNC13D* mutations were classified according to their functional impact. The group of *disruptive* mutations included the nonsense, frame shift and all splice errors, except c.952-1G>A and c.2626-1G>A (Santoro A, Aricò M, data not shown), which did not alter the frame; furthermore, the single nucleotide substitution c.1847A>G, based on our previous findings of its ability to induce a splicing error only in selected clones,³¹ was not included in the group of disruptive mutations. Based on this, the 84 patients were classified into three groups: *group 1*, defined by missense mutations only (n=17); *group 2*, including patients with one disruptive and one missense mutation (n=18); *group 3*, including patients with biallelic disruptive mutations (n=49).

In order to try to define differences between FHL2 and FHL3, data from the current cohort of patients with FHL3 were compared with available data from the previously reported cohort with FHL2.²⁸

Statistical analysis

Statistical significance of the differences between the ages at the disease onset and the quantitative evaluation of the cytotoxicity (in LU) between groups of patients with different mutations, was calculated by Student t test; to compare the differences in the proportions of CNS involvement, a χ^2 test was used. A p value 0.05 was considered as significant. Statistical analysis of the functional data comparing FHL3 and healthy donors was performed using one-way analysis of variance followed by Bonferroni's multiple comparison test, by GraphPad Prism 5 Software.

RESULTS

Study population

A total of 84 patients from 69 unrelated families were reported from the following reference centres: Florence, Italy, n=54; Hamburg, Germany, n=18; Stockholm, Sweden, n=12. They had been diagnosed between 1981 and 2009. Some of these patients had been previously reported as part of a single-centre series.^{29–33} Their ethnic origin was: Caucasian, n=58, Turkish, n=10, Asian, n=7, Hispanic, n=4, African, n=3 (not reported, n=2). Male: Female ratio was 1.2:1.

Presenting features

Main presenting features are summarised in table 1.

The median age at the diagnosis was 4.1 months, with a range of 1 day to 18.8 years (quartiles: 2.5, 4.1 and 18.4 months). Consanguinity was reported in 28/80 (35%) patients.

The current diagnostic criteria for HLH were fulfilled—that is they had at least five of the eight items proposed¹¹—by 58 of the 84 (69%) patients; of the remaining 26 patients, 15 had a positive family history, four had related parents, four had incomplete data and three fulfilled <5/8 criteria.

In particular, among those for whom the information was available, fever was present in 89% of patients, splenomegaly in 96%, bicytopenia in 88%, hypertriglyceridaemia and/or hypofibrinogenaemia in 95%, haemophagocytosis in 78%, hyperferritinaemia in 77% and low or absent NK cell activity in 98% (table 1).

A combination of fever, splenomegaly and thrombocytopenia was present in 65 of 77 patients (84%) evaluable for these three parameters; of these patients, 55 had information on ferritin level, which was raised in 39 (71%); otherwise, 59 of the 77 had information on fibrinogen level, which was reduced in 33 (56%); finally, all of the 77 had information on haemophagocytosis, which was present in 54 (69%) (figure 1).

CNS involvement was reported in 60% of the patients: 30/80 (38%) had neurological symptoms, 35/51 (69%) had 5 cells in the CSF, 22/31 (71%) elevated CSF protein and 15/25 (60%) MRI alterations.

UNC13D mutations

A total of 54 different mutations were observed in the 84 patients from 69 families. Nineteen were missense mutations, 14 deletions or insertions, 12 nonsense and nine splice errors (figure 2).

A total of 23 different mutations were observed at the homozygous state in 42 individuals from 36 unrelated families; their presenting features are summarised in table 2. The remaining 42 patients from 33 unrelated families were compound heterozygous. The most frequent mutations are described below.

The **c.2346_2349delGGAG** mutation, causing a frame shift at p.R782, was the most common mutation; it was identified in a total of 19 patients from 15 families, all Caucasian but one Turkish (not reported, n=1). In three homozygous patients the ages at the diagnosis were 2, 3 and 3 months; only one of them had CNS involvement. NK activity was reduced in the only patient analysed.

The **c.753+1G>T** mutation, resulting in a splice error, was found in 19 patients from 11 families (15%), nine Caucasian, one Asian and one Hispanic. Nine homozygous patients had a median age at the diagnosis of 5 months (range 3e12); four of them had CNS involvement. CD107a expression was absent in two and reduced in two patients analysed, NK activity was absent in three and reduced in three patients tested.

The **p.E616G** missense mutation, resulting from the c.1847A>G nucleotide change, was found in eight individuals from six families of Caucasian origin. Four patients carried the mutation at the homozygous state: age at diagnosis was 99 and 140 months in two unrelated subjects, and 6 and 226 months in two siblings. Three of the four had CNS involvement.

CD107a expression was reduced in the only patient tested; NK activity was reduced in two and absent in one of the three patients tested.

The p.**R928C** missense mutation (c.**2782C>T**) was identified in eight patients from seven unrelated families, six Caucasian and one Hispanic. This mutation falls in the C2B domain; its predicted impact is controversial, that is it may be tolerated according to SIFT, or potentially deleterious according to Polyphen. It was observed in three patients who also carried two additional mutations; one patient with this as second mutation had defective NK activity.

The nonsense mutation p.**R273X** (c.817C>T) was identified in six patients, including one pair of homozygous Caucasian siblings, both diagnosed at 2 months, and two additional heterozygous pairs, one Hispanic and one Caucasian. NK activity was absent in one patient tested.

The splice error c.**1389+1G>A** was identified in four unrelated patients (three Caucasian, one origin not reported). All were compound heterozygous with different frame shift (n=3) or nonsense (n=1) mutations.

Novel mutations

Fifteen novel mutations were identified in 15 patients; three were nonsense and five deletion/insertion mutations (figure 2, tables 2 and 3), while the remaining seven were missense mutations. The p.**I140T** missense mutation (c.419T>C) falls in the C2A domain and is predicted to be damaging. The p.**S398L** missense mutation, resulting from the c.1193C>T nucleotide change, falling outside the functional domains and predicted to be not tolerated, was identified in a 70-month Caucasian girl (UPN 257), with reduced NK activity. The p.**L647P** missense mutation (c.1940T>C) falls in the MHD1 domain and is predicted to be damaging. It was found in a 168-month-old male (UPN 397) presenting with typical FHL, reduced CD107 expression and NK activity was absent. The p.**R727Q** missense mutation (c.2180G>A) falls outside any functional domain and is predicted to be tolerated by SIFT and benign by Polyphen (score 0.353). It was observed in a compound heterozygous patient with defective degranulation. The p.**A859T** missense mutation (c.2575G>A) falls in the MHD2 domain and is predicted to be not tolerated. The p.**E1017K** missense mutation (c.3049G>A) falls in the C2B domain and is predicted to be not tolerated. The p.**L1058P** missense mutation (c.3173T>C) falls within the C-terminal domain and is predicted to be damaging. None of the missense mutations resulted in cryptic splice sites according to NNsplice software.

Analysis of control subjects

None of the following novel mutations were found in 100 healthy Caucasian control subjects: c.403insC, c.419T>C, c.1193C>T, c.1387C>T, c.1822del12, c.1940T>C, c.2057C>A, c.2180G>A, c.2212C>T, c.2437_2439delAACinsT, c.2477_2480delTCAC, c.2575G>A, c.3049G>A, c.3082delC, c.3173T>C.

Functional study

Granule release capacity was significantly reduced in 29 of the 30 patients tested. Of these patients, 17 had quantitative evaluation, analysing activated NK cells upon co-culture with K562, with a mean value of 14.5% of CD107a+ cells (SD 11.7, SE 2.8). This value was significantly inferior to that of healthy controls, both adults and infants ($p < 0.0001$).

Reduced or absent NK cytolytic activity was found in 44 of the 45 patients analysed (98%). Of these patients, 17 had quantitative evaluation of the LU, analysing activated NK cells against 721.221, with a mean value of 36.4 (SD 30.4, SE 7.3). This value was significantly inferior to that of healthy infant controls ($p < 0.005$).

Missense mutations falling in the functional domains

Overall, one-third of the mutations were missense: of these mutations, 11 were within the functional domains of the protein, while the remaining eight fell outside. Only three patients had biallelic missense mutations falling outside the domains: UPN 257 homozygous for c.1193C>T, UPN GE095 homozygous for c.1208C>T and UPN 483 heterozygous for c.175G>A and c.2180G>A. Their ages at diagnosis were 69.6, 2.6 and 0.7 months, respectively, with no significant difference from the remaining patients with missense mutations.

Genotype–phenotype correlations

Patients in group 1 (missense mutations, $n=17$), in group 2 (mixed mutations, $n=18$) and patients in group 3 (nonsense mutations, $n=49$) were compared to identify possible differences in their disease manifestations. Patients in group 3 were diagnosed at a significantly younger age than patients in groups 1 and 2 ($p < 0.0001$) (figure 3); moreover group 2 developed the disease at a significantly younger age than patients in group 1 ($p=0.01$).

No major differences in the symptoms present at diagnosis were detected between the patient groups. The combination of fever + splenomegaly + thrombocytopenia + ferritin elevation was present in 8 of the 13 (62%) evaluable patients in group 1 versus 27 of 33 (82%) in group 3 ($p=0.28$). The combination of fever + splenomegaly + thrombocytopenia was present in 13 of the 16 (81%) evaluable patients in group 1 vs 39 of 43 (91%) in group 3 ($p=0.58$). The frequency of CNS involvement also was not statistically different between the two groups (29/46, 63% in group 3 vs 10/17 59% in group 1; $p=0.98$).

Granule release capacity was significantly reduced in 29 of the 30 patients tested. Of these patients 17 had quantitative evaluation, analysing activated NK cells upon co-culture with K562, with a mean value of 14.5% of CD107a+ cells (SD 11.7, SE 2.8). This value was significantly inferior to that of healthy controls, both adults and infants ($p < 0.0001$). When patients of group 3 were analysed separately from those of groups 1 and 2, they did not differ from each other and both were significantly lower than healthy controls (figure 4A).

Reduced or absent NK cytolytic activity was found in 44 of the 45 patients analysed (98%). Of them 17 had quantitative evaluation of the LU, analysing activated NK cells against

721.221, with a mean value of 36.4 (SD 30.4, SE 7.3). This value was significantly inferior to that of healthy infant controls ($p < 0.005$). The mean LU value was not significantly lower in patients from group 3 than those in groups 1 and 2 (figure 4B).

Comparison of FHL2 and FHL3

The median age at the diagnosis of patients with FHL3 (4.1 months) was not significantly different from that previously observed in patients with FHL2 (3 months).²⁸ When the patients are classified according to the functional impact of the mutations, the age at diagnosis also is not significantly different for patients with missense (group 1) or with mixed mutations (group 2). Yet, when we focused on patients with biallelic disruptive mutations only, patients with FHL2 had a significantly younger age at the diagnosis, with a median age of 2 months compared with 3 months in patients with FHL3 (figure 5; $p = 0.001$).

CNS involvement was found in 49/81 (60%) patients, a frequency which is significantly higher than that reported in FHL2 ($n = 31/86$; 36%; $p = 0.001$).²⁸

DISCUSSION

This is the largest series of patients with FHL3 reported so far. Since *UNC13D* mutations have been found world wide, the predominance of Caucasian patients in this series only reflects the reporting bias of the European location of the contributing centres. However, owing to increasing immigration, a minority of families originating from Asia and Africa are also included.

FHL is usually considered a disease of early infancy. In this series, the median age at the diagnosis was 4.1 months, three-quarters of the cases being diagnosed within 18 months. When the patients are classified according to the functional impact of the mutations, patients with disruptive mutations developed the disease at a significantly younger age than patients with at least one allele bearing a non-disruptive mutation. This finding is in keeping with previous observations in FHL2, in which the median age at diagnosis (3 months) was slightly younger.²⁸ The age at diagnosis of patients with FHL2 or FHL3 having at least one missense mutation is comparable. Yet, when we focus on patients with biallelic disruptive mutations, those with FHL2 have a significantly younger median age at the diagnosis than those with FHL3 (2 vs 3 months). This might suggest that absence of perforin induces a disruption of the cytotoxic machinery which is even more deleterious than the priming impairment induced by a Munc13-4 defect. The onset of FHL is a conditional disease,³⁴ triggered by a viral pathogen. Since we have no evidence that patients with FHL3 had a later exposure to community-acquired pathogens, this might support the hypothesis that patients with complete perforin defect behave as though completely unable to cope with any common pathogen, whereas the degranulation defect induced by defective Munc13-4 may have some residual/redundant function, allowing residual defensive activity, at least against selected pathogens. Indeed, analysis of cytotoxic activity of activated NK cells, revealed a more striking defect of FHL2 than FHL3 NK cells when tested against various tumour cell lines or Epstein–Barr virus (EBV)-infected cells.³⁰ However, FHL3 NK cells, also tested upon activation, displayed a significantly lower degranulation capacity and cytotoxic activity than healthy controls (figure 4), while for other diseases (eg, FHL4, FHL5 and GS2)

the defects can be prevalently detected using resting NK cells.²⁵²⁶ This allows functional assays to be performed even using NK cells derived from patients studied many years ago, since these cells are polyclonal, activated, expanded in vitro and cryopreserved.

The age at the diagnosis of HLH has been reported to be usually consistent within individual families, although some exceptions are known.¹ Yet, in this series, two siblings were found to be homozygous for the c.1847A>G (p.E616G), a missense mutation which we previously documented to disrupt splicing and results in absence of the Munc13-4 protein.³¹ Both ultimately developed FHL3, although at a different age—namely, 6 months and 18.8 years. This is the most striking case of age discrepancy at the diagnosis of FHL3 in this series (figure 6). This observation further supports the hypothesis that an external trigger may be necessary to induce symptoms on the basis of a genetic predisposition. Whether or not additional disease-modifying genes are involved, we are not able to document, at present. All the above should be taken into account when counselling asymptomatic family members with documented genetic defect.

The diagnostic criteria for HLH were originally defined by the Histiocyte Society in 1994, and then revised in 2004.¹⁰¹¹ In this series, combination of five of the eight items—that is, the required standard for the diagnosis of HLH, was fulfilled in 69% of the patients. It was recently proposed that these criteria might be simplified.³⁵ In the attempt to contribute to this debate, we explored different combinations. The combination of fever, splenomegaly and thrombocytopenia, three clinical findings immediately available, was present in 84% of our patients. If, in addition, bone marrow aspiration, which is indicated to rule out the more frequent differential diagnosis ‘leukaemia’, demonstrates haemophagocytosis, the diagnosis is even more supported. In this series, the combination of these four findings was present in 69% of the patients also evaluated for bone marrow morphology. Alternatively, when the level of ferritin—an acute phase reactant widely available bedside—was investigated, it turned to be elevated in 71% of patients with fever, splenomegaly and thrombocytopenia. Thus, we suggest that the combination of fever, splenomegaly, and thrombocytopenia represents the initial clinical background to raise suspicion of FHL3; when associated with evidence of an increased ferritin level, these features may be considered as a very sensitive tool to address the diagnostic investigation even during the first few hours from admission. The sensitivity of this combination of clinical features for the diagnosis of other genotypic subsets remains to be evaluated.

It has been proposed that Munc13-4 deficiency may be associated with a higher rate of CNS manifestations. Although many proteins of the Munc family have specific roles in the CNS, Munc 13-4 is not expressed in the brain,³⁶ rendering a specific impact of the defective protein unlikely in the pathogenesis of CNS disease in FHL3. CNS involvement was found in 60% of the patients. While only one-third of the cases had neurological symptoms at the diagnosis, 69% had 5 cells in the CSF, 71% had elevated CSF protein and 60% had MR alterations. These data suggest that a Munc13-4 defect induces a higher proportion of CNS involvement than a perforin defect, with only 36% of patients with FHL2 showing CNS involvement.²⁸³⁷

UNC13D mutations are scattered over the entire gene, without any apparent clustering. This confirms that the strategy of analysis cannot rely on any preferential region(s).³⁸ A total of 54 different mutations were collected, suggesting that no founder effect is evident in FHL3. In contrast to FHL2, we did not observe any correlation between ethnic groups and specific mutations. It may be worth mentioning that the c.1596+1G>C mutation was reported to be the most common *UNC13D* mutation in Japan, accounting for 70% of the patients.³⁹ In addition, recently Yoon *et al* reported that c.754-1G>C accounts for the majority of *UNC13D* mutations (58%) in Korean patients with FHL3.⁴⁰ These mutations were not found in our series, which did not include any Japanese or Korean patients. In this series we report 15 novel mutations. Two mutations, c.2346_2349delGGAG and c.753+1G>T, accounted for 26 of the 69 unrelated families (38%). As already reported, 46% of patients with FHL3 have at least one mutation responsible for a splicing error,³¹ as expected because of the complex structure of *UNC13D*, with its high number of exons, which is a well-known predisposing factor for the occurrence of this type of aberration.

Overall, one-third of the mutations were missense, including seven novel ones. Their pathogenic role may be questionable. The missense mutation c.1193C>T (p.S398L) was found at the homozygous state in a Caucasian patient who presented at the age of 70 months. Of the four patients with homozygous p.E616G mutations, three presented at 99, 140 and 226 months. We have previously proved that this mutation is associated with a splice error and protein defect.³¹ Missense mutations p.L647P, p.R608P and p.R928P were associated with reduced cytotoxic function, protein expression and later onset, suggesting a hypomorphic effect.

In patients with suspected FHL appropriate treatment should be started promptly, since about 20% of the patients may die within the first few weeks despite specific treatment. Although mutation analysis remains the ‘gold standard’ for diagnosis of FHL, results are not readily available. We found defective degranulation in almost all of the evaluated patients with FHL3. We therefore recommend that patients with a robust clinical suspicion based on evidence of fever, splenomegaly, thrombocytopenia and haemophagocytosis, or high ferritin level, should have a functional screening including perforin expression and cytotoxic lymphocyte degranulation assays by flow cytometry^{30,41,42} at national reference laboratories. All patients fulfilling the diagnostic pattern and with a degranulation defect should be considered eligible for immediate empirical treatment, followed by directed mutation analysis to support an indication for haematopoietic stem cell transplantation as a curative therapeutic programme. Given the high frequency of mutations leading to splicing errors, the strategy of analysis must include the RNA study.

Acknowledgments

Funding This work was partly supported by the following sources: European Union 7th Framework Program under grant agreement No HEALTH-F2-2008-201461; “Antonio Pinzino - Associazione per la Ricerca sulle Sindromi Emofagocitiche (ARSE)”; “Noi per Voi per il Meyer Onlus”; Italian Ministry of Health, Bando “Malattie Rare 2008”; A.O.U. Meyer; Swedish Children’s Cancer Foundation, the Swedish Research Council, the Cancer and Allergy Foundation of Sweden, the Swedish Cancer Foundation, the Karolinska Institutet and the Stockholm County Council.

REFERENCES

1. Aricò M, Janka G, Fischer A, Henter JI, Blanche S, Elinder G, Martinetti M, Rusca MP. Hemophagocytic lymphohistiocytosis. Report of 122 children from the International Registry. FHL Study Group of the Histiocyte Society. *Leukemia*. 1996; 10:197–203. [PubMed: 8637226]
2. Henter JI, Samuelsson-Horne A, Aricò M, Egeler RM, Elinder G, Filipovich AH, Gadner H, Imashuku S, Komp D, Ladisch S, Webb D, Janka G. Histiocyte Society. Treatment of hemophagocytic lymphohistiocytosis with HLH-94 immunochemotherapy and bone marrow transplantation. *Blood*. 2002; 100:2367–73. [PubMed: 12239144]
3. Mahlaoui N, Ouachee-Chardin M, de Saint Basile G, Neven B, Picard C, Blanche S, Fischer A. Immunotherapy of familial hemophagocytic lymphohistiocytosis with antithymocyte globulins: a single-center retrospective report of 38 patients. *Pediatrics*. 2007; 120:e622–8. [PubMed: 17698967]
4. Jabado N, de Graeff-Meeder ER, Cavazzana-Calvo M, Haddad E, Le Deist F, Benkerrou M, Dufourcq R, Caillat S, Blanche S, Fischer A. Treatment of familial hemophagocytic lymphohistiocytosis with bone marrow transplantation from HLA genetically nonidentical donors. *Blood*. 1997; 90:4743–8. [PubMed: 9389690]
5. Horne A, Janka G, Egeler MR, Gadner H, Imashuku S, Ladisch S, Locatelli F, Montgomery SM, Webb D, Winiarski J, Filipovich AH, Henter JI, Histiocyte Society. Haematopoietic stem cell transplantation in haemophagocytic lymphohistiocytosis. *Br J Haematol*. 2005; 129:622–30. [PubMed: 15916685]
6. Baker KS, Filipovich AH, Gross TG, Grossman WJ, Hale GA, Hayashi RJ, Kamani NR, Kurian S, Kapoor N, Ringdén O, Eapen M. Unrelated donor hematopoietic cell transplantation for hemophagocytic lymphohistiocytosis. *Bone Marrow Transplant*. 2008; 42:175–80. [PubMed: 18454181]
7. Cooper N, Rao K, Goulden N, Webb D, Amrolia P, Veys P. The use of reduced-intensity stem cell transplantation in haemophagocytic lymphohistiocytosis and Langerhans cell histiocytosis. *Bone Marrow Transplant*. 2008; 42(Suppl 2):S47–50. [PubMed: 18978744]
8. Cesaro S, Locatelli F, Lanino E, Porta F, Di Maio L, Messina C, Prete A, Ripaldi M, Maximova N, Giorgiani G, Rondelli R, Aricò M, Fagioli F. Hematopoietic stem cell transplantation for hemophagocytic lymphohistiocytosis: a retrospective analysis of data from the Italian Association of Pediatric Hematology Oncology (AIEOP). *Haematologica*. 2008; 93:1694–701. [PubMed: 18768529]
9. Aricò M, Allen M, Brusa S, Clementi R, Pende D, Maccario R, Moretta L, Danesino C. Haemophagocytic lymphohistiocytosis: proposal of a diagnostic algorithm based on perforin expression. *Br J Haematol*. 2002; 119:180–8. [PubMed: 12358924]
10. Henter JI, Elinder G, Ost A. Diagnostic guidelines for hemophagocytic lymphohistiocytosis. The FHL Study Group of the Histiocyte Society. *Semin Oncol*. 1991; 18:29–33. [PubMed: 1992521]
11. Henter JI, Horne A, Aricò M, Egeler RM, Filipovich AH, Imashuku S, Ladisch S, McClain K, Webb D, Winiarski J, Janka G. HLH-2004: diagnostic and therapeutic guidelines for Hemophagocytic lymphohistiocytosis. *Pediatr Blood Cancer*. 2007; 48:124–31. [PubMed: 16937360]
12. Perez N, Virelizier JL, Arenzana-Seisdedos F, Fischer A, Griscelli C. Impaired natural killer activity in lymphohistiocytosis syndrome. *J Pediatr*. 1984; 104:569–73. [PubMed: 6368780]
13. Aricò M, Nespoli L, Maccario R, Montagna D, Bonetti F, Caselli D, Burgio GR. Natural cytotoxicity impairment in familial haemophagocytic lymphohistiocytosis. *Arch Dis Child*. 1988; 63:292–6. [PubMed: 3355209]
14. Schneider EM, Lorenz I, Muller-Rosenberger M, Steinbach G, Kron M, Janka-Schaub GE. Hemophagocytic lymphohistiocytosis is associated with deficiencies of cellular cytolysis but normal expression of transcripts relevant to killer-cell-induced apoptosis. *Blood*. 2002; 100:2891–8. [PubMed: 12351400]
15. Farquhar JW, Claireaux AE. Familial haemophagocytic reticulosis. *Arch Dis Child*. 1952; 27:519–25. [PubMed: 13008468]

16. Ohadi M, Lalloz MR, Sham P, Zhao J, Dearlove AM, Shiach C, Kinsey S, Rhodes M, Layton DM. Localization of a gene for familial hemophagocytic lymphohistiocytosis at chromosome 9q21.3-22 by homozygosity mapping. *Am J Hum Genet.* 1999; 64:165–71. [PubMed: 9915955]
17. Dufourcq-Lagelouse R, Jabado N, Le Deist F, Stéphan JL, Souillet G, Bruin M, Vilmer E, Schneider M, Janka G, Fischer A, de Saint Basile G. Linkage of familial hemophagocytic lymphohistiocytosis to 10q21-22 and evidence for heterogeneity. *Am J Hum Genet.* 1999; 64:172–9. [PubMed: 9915956]
18. Stepp SE, Dufourcq-Lagelouse R, Le Deist F, Bhawan S, Certain S, Mathew PA, Henter JI, Bennett M, Fischer A, de Saint Basile G, Kumar V. Perforin gene defects in familial hemophagocytic lymphohistiocytosis. *Science.* 1999; 286:1957–9. [PubMed: 10583959]
19. Clementi R, zur Stadt U, Savoldi G, Varotto S, Conter V, De Fusco C, Notarangelo LD, Schneider M, Klersy C, Janka G, Danesino C, Aricò M. Six novel mutations in the PRF1 gene in children with haemophagocytic lymphohistiocytosis. *J Med Genet.* 2001; 38:643–6. [PubMed: 11565555]
20. Risma KA, Frayer RW, Filipovich AH, Sumegi J. Aberrant maturation of mutant perforin underlies the clinical diversity of hemophagocytic lymphohistiocytosis. *J Clin Invest.* 2006; 116:182–92. [PubMed: 16374518]
21. Ueda I, Morimoto A, Inaba T, Yagi T, Hibi S, Sugimoto T, Sako M, Yanai F, Fukushima T, Nakayama M, Ishii E, Imashuku S. Characteristic perforin gene mutations of haemophagocytic lymphohistiocytosis patients in Japan. *Br J Haematol.* 2003; 121:503–10. [PubMed: 12716377]
22. Feldmann J, Le Deist F, Ouachée-Chardin M, Certain S, Alexander S, Quartier P, Haddad E, Wulffraat N, Casanova JL, Blanche S, Fischer A, de Saint Basile G. Functional consequences of perforin gene mutations in 22 patients with familial haemophagocytic lymphohistiocytosis. *Br J Haematol.* 2002; 117:965–72. [PubMed: 12060139]
23. Feldmann J, Callebaut I, Raposo G, Certain S, Bacq D, Dumont C, Lambert N, Ouachee-Chardin M, Chedeville G, Tamary H, Minard-Colin V, Vilmer E, Blanche S, Le Deist F, Fischer A, de Saint Basile G. Munc13-4 is essential for cytolytic granules fusion and is mutated in a form of familial hemophagocytic lymphohistiocytosis (FHL3). *Cell.* 2003; 115:461–73. [PubMed: 14622600]
24. zur Stadt U, Schmidt S, Kasper B, Beutel K, Diler AS, Henter JI, Kabisch H, Schneppenheim R, Nürnberg P, Janka G, Hennies HC. Linkage of familial hemophagocytic lymphohistiocytosis (FHL) type-4 to chromosome 6q24 and identification of mutations in syntaxin 11. *Hum Mol Genet.* 2005; 14:827–34. [PubMed: 15703195]
25. Bryceson YT, Rudd E, Zheng C, Edner J, Ma D, Wood SM, Bechensteen AG, Boelens JJ, Celkan T, Farah RA, Hultenby K, Winiarski J, Roche PA, Nordenskjöld M, Henter JI, Long EO, Ljunggren HG. Defective cytotoxic lymphocyte degranulation in syntaxin-11 deficient familial hemophagocytic lymphohistiocytosis (FHL4) patients. *Blood.* 2007; 110:1906–15. [PubMed: 17525286]
26. zur Stadt U, Rohr J, Seifert W, Koch F, Grieve S, Pagel J, Strauss J, Kasper B, Nürnberg G, Becker C, Maul-Pavicic A, Beutel K, Janka G, Griffiths G, Ehl S, Hennies HC. Familial Hemophagocytic Lymphohistiocytosis Type 5 (FHL-5) Is Caused by Mutations in Munc18-2 and Impaired Binding to Syntaxin 11. *Am J Hum Genet.* 2009; 85:482–92. [PubMed: 19804848]
27. Côte M, Ménager MM, Burgess A, Mahlaoui N, Picard C, Schaffner C, Al-Manjomi F, Al-Harbi M, Alangari A, Le Deist F, Gennery AR, Prince N, Cariou A, Nitschke P, Blank U, El-Ghazali G, Ménasché G, Latour S, Fischer A, de Saint Basile G. Munc18-2 deficiency causes familial hemophagocytic lymphohistiocytosis type 5 and impairs cytotoxic granule exocytosis in patient NK cells. *J Clin Invest.* 2009; 119:3765–73. [PubMed: 19884660]
28. Trizzino A, zur Stadt U, Ueda I, Risma K, Janka G, Ishii E, Beutel K, Sumegi J, Cannella S, Pende D, Mian A, Henter JI, Griffiths G, Santoro A, Filipovich A, Aricò M, Histocyte Society HLH Study group. Genotype-phenotype study of familial haemophagocytic lymphohistiocytosis due to perforin mutations. *J Med Genet.* 2008; 45:15–21. [PubMed: 17873118]
29. Santoro A, Cannella S, Bossi G, Gallo F, Trizzino A, Pende D, Dieli F, Bruno G, Stinchcombe JC, Micalizzi C, De Fusco C, Danesino C, Moretta L, Notarangelo LD, Griffiths GM, Aricò M. Novel Munc13-4 mutations in children and young adult patients with haemophagocytic lymphohistiocytosis. *J Med Genet.* 2006; 43:953–60. [PubMed: 16825436]

30. Marcenaro S, Gallo F, Martini S, Santoro A, Griffiths GM, Aricò M, Moretta L, Pende D. Analysis of natural killer-cell function in familial Hemophagocytic lymphohistiocytosis (FHL): defective CD107a surface expression heralds Munc13-4 defect and discriminates between genetic subtypes of the disease. *Blood*. 2006; 108:2316–23. [PubMed: 16778144]
31. Santoro A, Cannella S, Trizzino A, Bruno G, De Fusco C, Notarangelo LD, Pende D, Griffiths GM, Aricò M. Mutations affecting mRNA splicing are the most common molecular defect in patients with familial hemophagocytic lymphohistiocytosis type 3. *Haematologica*. 2008; 93:1086–90. [PubMed: 18492689]
32. Zur Stadt U, Beutel K, Kolberg S, Schneppenheim R, Kabisch H, Janka G, Hennies HC. Mutation spectrum in children with primary hemophagocytic lymphohistiocytosis: molecular and functional analyses of PRF1, UNC13D, STX11, and RAB27A. *Hum Mutat*. 2006; 27:62–8. [PubMed: 16278825]
33. Rudd E, Bryceson YT, Zheng C, Edner J, Wood SM, Ramme K, Gavhed S, Gürgey A, Hellebostad M, Bechensteen AG, Ljunggren HG, Fadeel B, Nordenskjöld M, Henter JI. Spectrum, and clinical and functional implications of UNC13D mutations in familial haemophagocytic lymphohistiocytosis. *J Med Genet*. 2008; 45:134–41. [PubMed: 17993578]
34. Crozat K, Hoebe K, Ugolini S, Hong NA, Janssen E, Rutschmann S, Mudd S, Sovath S, Vivier E, Beutler B. Jinx, an MCMV susceptibility phenotype caused by disruption of Unc13d: a mouse model of type 3 familial hemophagocytic lymphohistiocytosis. *J Exp Med*. 2007; 204:853–63. [PubMed: 17420270]
35. Filipovich AH. Hemophagocytic lymphohistiocytosis (HLH) and related disorders. *Hematology Am Soc Hematol Educ Program*. 2009:127–31. [PubMed: 20008190]
36. Ménasché G, Feldmann J, Fischer A, de Saint Basile G. Primary hemophagocytic syndromes point to a direct link between lymphocyte cytotoxicity and homeostasis. *Immunol Rev*. 2005; 203:165–79. [PubMed: 15661029]
37. Horne AC, Trottestam H, Aricò M, Egeler RM, Filipovich AH, Gadner H, Imashuku S, Ladisch S, Webb D, Janka G, Henter JI, Histiocyte Society. Frequency and spectrum of central nervous system involvement in 193 children with haemophagocytic lymphohistiocytosis. *Br J Haematol*. 2008; 140:327–35. [PubMed: 18076710]
38. Cetica V, Pende D, Griffiths GM, Aricò M. Molecular basis of familial hemophagocytic lymphohistiocytosis. *Haematologica*. 2010; 95:538–41. [PubMed: 20378576]
39. Ueda I, Ishii E, Morimoto A, Ohga S, Sako M, Imashuku S. Correlation between phenotypic heterogeneity and gene mutational characteristics in familial hemophagocytic lymphohistiocytosis (FHL). *Pediatr Blood Cancer*. 2006; 46:482–8. [PubMed: 16365863]
40. Yoon HS, Kim HJ, Yoo KH, Sung KW, Koo HH, Kang HJ, Shin HY, Ahn HS, Kim JY, Lim YT, Bae KW, Lee KO, Shin JS, Lee ST, Chung HS, Kim SH, Park CJ, Chi HS, Im HJ, Seo JJ. UNC13D is the predominant causative gene with recurrent splicing mutations in Korean patients with familial hemophagocytic lymphohistiocytosis. *Haematologica*. 2010; 95:622–6. [PubMed: 20015888]
41. Wheeler RD, Cale CM, Cetica V, Aricò M, Gilmour KC. A novel assay for investigation of suspected familial haemophagocytic lymphohistiocytosis. *Br J Haematol*. 2010; 150:727–30. [PubMed: 20618327]
42. Kogawa K, Lee SM, Villanueva J, Marmer D, Sumegi J, Filipovich AH. Perforin expression in cytotoxic lymphocytes from patients with hemophagocytic lymphohistiocytosis and their family members. *Blood*. 2002; 99:61–6. [PubMed: 11756153]

Figure 1.

Distribution of the frequency of some diagnostic parameters for haemophagocytic lymphohistiocytosis and pattern of their association.

Figure 2.

Details of 54 different *UNC13D* mutations observed in 84 patients with familial haemophagocytic lymphohistiocytosis type 3 (FHL3) from 69 families. Mutations are spread over the entire gene. Disruptive mutations are indicated in bold.

Figure 3.

Age at onset of familial haemophagocytic lymphohistiocytosis type 3 (FHL3) depends on the functional impact of the *UNC13D* mutations. Patients with two disruptive mutations had a significantly younger age at diagnosis than patients with two missense mutations. Each point represents one person, lines indicate mean values.

Figure 4.

Impaired degranulation and cytotoxicity by activated NK cells from patients with familial haemophagocytic lymphohistiocytosis type 3 (FHL3). Polyclonal activated NK cell populations derived from healthy adult and infant donors, and from patients with FHL3, considered separately as group 1+2 and group 3, were analysed for surface expression of CD107a after stimulation with K562 (data are expressed as percentage ΔCD107a, panel A) and for cytolytic activity against 721.221 (data are expressed as LU at 30% lysis, panel B).

Each point represents one person, lines indicate mean values. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.001$; NS, not significant.

Figure 5.

Age at onset of familial haemophagocytic lymphohistiocytosis in patients with two disruptive mutations is significantly younger in patients with FHL type 2 (FHL2) than in patients with FHL type 3 (FHL3). Each point represents one person, lines indicate mean values.

Figure 6.

Comparison of age at onset in 14 families with familial haemophagocytic lymphohistiocytosis type 3 (FHL3) and at least two affected siblings.

Table 1

Characteristics of 84 patients with familial haemophagocytic lymphohistiocytosis and *UNC13D* mutations

Characteristics	Number/total (%)
Age	
►Median	4.1 Months
►Range	At birth to 18.8 years
►Quartiles	2.5, 4.1, 18.4 Months
Consanguinity	28/80 (35)
Fever	72/81 (89)
Splenomegaly	78/81 (96)
Central nervous system disease	49/81 (60)
Anaemia	55/71 (77)
Neutropenia	53/75 (71)
Thrombocytopenia	72/77 (94)
Hypofibrinogenaemia	37/60 (62)
Hypertriglyceridaemia	49/63 (78)
Hyperferritinaemia	43/56 (77)
Haemophagocytosis	63/81 (78)
Defective NK activity	44/45 (98)

Main clinical features and initial laboratory evaluation of 42 patients with familial haemophagocytic lymphohistiocytosis type 3 and homozygous mutations

Table 2

Mutation	UPN	Ethnic group	Age at diagnosis (months)	Fever	Splenomegaly	CNS disease	Anaemia	Neutropenia	Thrombocytopenia	Hyperferritinaemia	Hypertriglyceridaemia	Hypofibrinogenaemia	CD1107 expression [‡]	NK activity [§]	Munc 13-4 expression [¶]
c.214delC (p.P72fs)	423	Asian	3	–	–	–	NA	NA	NA	NA	NA	NA	Reduced	NA	NA
c.247C>T (p.R83X)	SWE01	Caucasian	0	–	+	–	+	+	+	–	–	–	NA	NA	NA
c.441delA (p.P147fs)	336 [‡]	Caucasian	4	+	+	+	+	+	+	+	+	+	Absent	Absent	Absent
c.532delC (p.Q178fs)	3	Caucasian	3	+	+	–	–	+	+	NA	+	+	NA	Reduced	NA
c.627delT (p.T209fs)	GE123	Turkish	21	+	+	+	NA	+	+	+	+	+	NA	NA	NA
c.640C>T (p.R214X)	SWE06 [*]	Turkish	2	+	+	–	+	+	+	+	+	–	NA	NA	NA
	SWE07 [*]	Turkish	2	+	+	–	+	+	+	+	+	NA	NA	NA	NA
c.753+1G>T	173	Caucasian	6	+	+	–	+	+	+	–	–	+	NA	Absent	NA
	180 [*] [‡]	Caucasian	3	+	+	–	+	–	+	–	–	+	Absent	Absent	Absent
	229 [*] [‡]	Caucasian	4	+	+	–	+	+	+	NA	NA	+	NA	NA	NA
	285	Caucasian	3	+	+	+	–	–	+	NA	NA	NA	Absent	Absent	Absent
	363	Hispanic	12	+	+	+	+	+	+	+	+	–	NA	NA	NA
	419	Caucasian	11	+	+	–	NA	NA	NA	NA	NA	NA	Reduced	Reduced	NA
	GE085		3	+	+	+	+	–	+	+	–	+	NA	Reduced	NA
	SWE12	Caucasian	7	+	+	+	+	+	+	+	+	+	Reduced	Reduced	NA
	SWE13	Asian	NA	+	+	–	NA	NA	NA	–	+	–	NA	NA	NA
c.817C>T (p.R273X)	46 [*]	Caucasian	2	–	+	–	NA	+	+	NA	–	+	NA	NA	NA
	159 [*]	Caucasian	2	+	+	–	+	+	+	–	+	–	NA	Absent	NA
c.952-1G>A	390	Asian	3	+	+	–	+	+	+	+	+	–	Reduced	NA	NA
c.1055+1G>A	GE047	African	19	+	+	+	+	–	+	+	+	–	NA	NA	NA
c.1145G>A (p.W382X)	SWE08	Asian	2	+	+	+	+	+	+	+	+	+	Reduced	NA	NA
c.1193C>T (p.S398L)	257	Caucasian	70	+	+	–	–	–	+	NA	NA	–	NA	Reduced	NA
c.1208T>C (p.L403P)	GE095	Turkish	3	+	+	–	+	+	+	+	+	+	NA	Absent	NA
c.1225delC (p.L409fs)	187	Caucasian	3	+	+	–	+	+	+	NA	+	+	NA	Absent	NA
c.1822_1833del12 (p.V608fs)	293	African	2	–	+	–	+	+	+	+	+	+	Absent	Absent	Reduced
c.1847A>G (p.E616G)	197 [*]	Caucasian	6	+	+	–	+	–	–	NA	NA	NA	NA	NA	NA
	198 [*]	Caucasian	226	+	+	+	+	–	+	+	+	+	NA	Absent	NA
	249	Caucasian	99	+	+	+	–	+	+	–	+	–	NA	Reduced	Absent

Mutation	UPN	Ethnic group	Age at diagnosis (months)	Fever	Splenomegaly	CNS disease	Anaemia	Neutropenia	Thrombocytopenia	Hyperferritinaemia	Hypertriglyceridaemia	Hypofibrinogenaemia	CD107 expression [‡]	NK activity [§]	Munc 13-4 expression [¶]
c.1940T>C (p.L647P) c.2039C>G (p.R680P) c.2057C>A (p.S686X) c.2346_2349delGGAG (p. R782fs)	484	Caucasian	140	+	+	+	+	+	+	NA	NA	NA	Reduced	Reduced	Absent
	397	Caucasian	168	+	+	+	+	+	+	+	+	–	Reduced	Absent	NA
	132	Caucasian	83	+	+	+	+	+	+	+	+	+	NA	Reduced	NA
	442	African	5	+	+	–	+	+	+	+	+	+	Reduced	Reduced	NA
c.2626-1G>A	GE242	Caucasian	2	+	+	–	+	+	+	+	+	–	NA	NA	NA
	GE516	Turkish	3	+	+	–	+	–	+	+	NA	NA	NA	NA	NA
	SWE04	Caucasian	3	+	+	+	+	NA	+	NA	NA	NA	NA	Reduced	NA
	SWE02*	Asian	168	–	+	–	+	+	+	+	+	NA	Reduced	Reduced	NA
c.2783G>C (p.R928P)	SWE03*	Asian	120	+	+	+	+	+	+	+	–	–	NA	Reduced	NA
	SWE05*	Turkish	1	+	+	–	NA	NA	+	+	+	+	NA	NA	NA
	SWE09*	Turkish	36	+	+	–	+	–	+	–	+	NA	NA	NA	NA
	520	Indian	6	+	+	+	+	+	+	+	+	–	NA	NA	NA
c.3193C>T (p.R1065X)	GE101	Caucasian	17	+	+	+	+	+	+	+	+	–	NA	Reduced	NA
	GE105	Turkish	6	+	+	+	+	+	+	+	+	+	NA	Absent	NA

* Siblings.

[‡]These patients also carried the c.175G>A (p.A59T) mutation.

[‡]In the degranulation assays, CD107 expression was considered ‘absent’ if <5% or 10% in resting or activated NK cells, respectively; ‘reduced’ if significantly (p<0.05) lower than controls.

[§]In the cytotoxicity assays, NK activity was considered ‘absent’ if <5% or 30% lysis at the highest E:T ratio examined using either resting or activated NK cells, respectively; ‘reduced’ if significantly (p<0.05) lower than controls.

[¶]Munc13-4 was tested by Western blot.

Novel mutations are in bold.

CNS, central nervous system; NA, not available; UPN, unique patient number.

Table 3

Main clinical features and initial laboratory evaluation of 11 patients with familial haemophagocytic lymphohistiocytosis with compound heterozygous novel mutations

Allele 1 mutation	Allele 2 mutation	UPN	Ethnic group	Age at diagnosis (months)	Fever	Splenomegaly	CNS disease	Anaemia	Neutropenia	Thrombocytopenia	Hyperferritinaemia	Hypertriglyceridaemia	Hypofibrinogenaemia	CD107 expression [†]	NK activity [‡]	Munc 13-4 expression [§]
c.403InsC (p.Y135Fs)	c.627delT (p.T209Fs)	481	Caucasian	28	+	+	+	+	+	+	+	–	–	Reduced	Reduced	NA
c.419T>C (p.L140T)	c.2782C>T (p.R928C)	349	Hispanic	106	+	+	–	+	+	NA	+	+	NA	NA	NA	NA
c.1387C>T (p.Q463X)	c.753+1G>T	183	Caucasian	3	+	+	–	+	–	+	NA	NA	NA	NA	NA	NA
c.1387C>T (p.Q463X)	c.753+1G>T	182	Caucasian	1	+	+	–	+	–	+	NA	NA	NA	NA	NA	NA
c.2180G>A (p.R727Q)	c.175G>A (p.A59T)	483	Caucasian	1	–	–	–	–	+	+	–	+	+	Reduced	NA	NA
c.2212C>T (p.Q738X)	c.3173T>C (p.L1058P)	GE 268	Caucasian	6	+	+	–	+	+	+	+	–	–	Reduced	Reduced	NA
c.2437_2439delAAHisT (p.N813Fs)	c.2346_2349delGGAG (p.R782Fs)	472	Caucasian	1	+	+	–	–	–	+	+	+	NA	NA	NA	NA
c.2477_2480delTCAC (p.L826Fs)	c.2346_2349delGGAG (p.R782Fs)	532	NA	3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
c.2575G>A (p.A859T)	c.1225delC (p.L409Fs)	245*	Caucasian	96	–	–	+	+	–	+	NA	NA	NA	Absent	Absent	NA
c.3049G>A (p.E1017K)	c.2782C>T (p.R928C)	208	Caucasian	58	+	+	NA	+	–	+	+	+	+	NA	Absent	NA
c.3082delC (p.L1028Fs)	c.2782C>T (p.R928C)	277	Caucasian	119	+	+	–	–	+	+	NA	NA	NA	Reduced	Absent	Absent

* This patient also carried the c.2782C>T (p.R928C) mutation.

[†] In the degranulation assays, CD107 expression was considered ‘absent’ if <5% or 10% in resting or activated NK cells, respectively; ‘reduced’ if significantly (p<0.05) lower than controls.

[‡] In the cytotoxicity assays, NK activity was considered ‘absent’ if <5% or 30% lysis at the highest E:T ratio examined using either resting or activated NK cells, respectively; ‘reduced’ if significantly (p<0.05) lower than controls.

[§] Munc 13-4 was tested by Western blot.

Novel mutations are in bold.

CNS, central nervous system; NA, not available; UPN, unique patient number.