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GENOTYPE-PHENOTYPE STUDY OF FAMILIAL HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS TYPE 3

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ABSTRACT (253 words)

Background: Mutations of *UNC13D* are causative for FHL3 (OMIM 608898). We present a genotype-phenotype study of 84 FHL3 patients.
Methods: A consortium of 3 countries planned to pool in a common database data on presenting features and mutations from individual patients with biallelic *UNC13D* mutations.
Results: 84 FHL3 patients (median age: 4.1 months) were reported from Florence, Italy (n=54), Hamburg, Germany (n=18), Stockholm, Sweden (n=12). Their ethnic origin was: Caucasian, n=57, Turkish, n=10, Asian, n=7, Hispanic, n=4, African, n=3 (not reported, n=3). Thrombocytopenia was present in 96%, splenomegaly in 95%, fever in 89%. Central nervous system was involved in 49/81 (60%) patients versus 36% in FHL2 (p=0.001). The combination of fever, splenomegaly, thrombocytopenia and hyperferritinemia was present in 71%. CD107a expression, NK activity and Munc13-4 protein expression were absent or reduced in all but one of the evaluated patients.
We observed 54 different mutations, including 15 novel ones: 19 missense, 14 deletions or insertions, 12 nonsense, 9 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 FHL2 patients (p=0.001).
Conclusion. *UNC13D* mutations are scattered over the gene. Ethnic-specific mutations were not identified. CNS involvement is more frequent 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 hyperferritinemia appears to be the most easily and frequently recognized clinical pattern and their association with defective granule release assay may herald FHL3.

INTRODUCTION

Hemophagocytic lymphohistiocytosis (HLH) is a genetically heterogeneous disorder characterized by a hyperinflammatory syndrome with fever, hepatosplenomegaly, cytopenia, and frequently also central nervous system 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, cyclosporine, etoposide, anti-thymocyte globuline, can obtain transient disease control.[2,3] So far, haematopoietic stem cell transplantation appears to be the only curative treatment. [4-8]

Differential diagnosis of HLH may be difficult.[9] To 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 et al. 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] Very recently, zur Stadt et al. have allocated a novel FHL type, FHL-5 (OMIM 613101), to a 1 Mb region on chromosome 19p using high resolution SNP genotyping in eight unrelated FHL patients from consanguineous families. They have 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 FHL-5 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. Hemophagocytic Lymphohistiocytosis was defined by the diagnostic criteria established by the Histiocyte Society.[10,11] Central nervous system (CNS) involvement was defined as the presence of at least one of the following items: neurologic symptoms, CSF pleocytosis (≥5 cells/mm^3), elevated CSF protein (≥30 mg/dl), MRI alteration. All data were stored in a common database and analysed. The completeness of the data was: ethnicity, 95%; consanguinity, 95%; age at diagnosis, 98%; persistent fever, 96%; splenomegaly, 96%; central nervous system (CNS) disease, 95%; haemoglobin 85%; neutrophils, 89%; platelets, 92%; triglycerids, 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 IRB 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.[1,19,28-30] The 32 coding exons and exon-intron boundaries of *Munc13-4* gene were amplified and directly sequenced, in both directions, with the BigDye® Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). Amplification reactions were performed with 60 ng of DNA, 10 ng of each

primer, 200 µM dNTPs, 1x PCR reaction buffer, and 2.5 U Taq polymerase in a final volume of 25µl; primers are available upon request. Sequences obtained using an ABI Prism® 3130XL Sequence Detection System (Applied Biosystems) were analyzed 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 will 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 informations and defines a substitution to be “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/seq_tools/splice).

Functional analyses

Peripheral blood mononuclear cells (PBMC) from FHL3 patients and healthy donors were isolated by Ficoll gradient centrifugation. NK cells were also purified using the RosetteSep method (StemCell Technologies, Vancouver, British Columbia, Canada) following

manufacturer's instructions. NK cells were cultured on irradiated feeder cells in the presence of 2 µg/mL phytohemagglutinin (Sigma-Aldrich, Irvine, UK) and 100U/mL rIL-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, PBMC 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 FHL3 patients.[30] E:T ratios ranging from 100:1 to 1:1 were used for PBMC as effector cells, while from 8:1 to 0.5:1 for activated NK. Lytic units (LU) at 30% lysis were calculated. PBMC and activated NK cells were also tested in 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 mAb was added prior to incubation of 3 hours at 37° C in 5% CO2. Thereafter, the cells were stained with anti-CD56-APC and anti-CD3-PerCP mAb and analysed by flow cytometry (FACSCalibur, Becton Dickinson). Surface expression of CD107a was assessed in the CD3$^-$ CD56$^+$ cells. Results are reported as ΔCD107a (i.e. % CD107a$^+$ cells of stimulated - % 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, frameshift and all splice errors, except c.952-1G>A and c.2626-1G>A (Santoro A, Aricò M., data not shown), that did not alter the frame; furthermore, the single nucleotide substitution c.1847A>G, based on our previous findings of its capacity 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 3 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 cytoxicity (in lytic units) between groups of patients with different mutations, was calculated by Student t test; to compare the differences in the proportions of CNS involvement, Chi-Square test was used. P value ≤0.05 was considered as significant. Statistical analysis of the functional data comparing FHL3 and healthy donors was performed using one-way ANOVA 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 single-centre series.[29-33] The ethnic origin was as follows: 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 summarized 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 of 80 (35%) patients.

The current diagnostic criteria for HLH were fulfilled – i.e. they had at least five of the eight items proposed [11] – by 58 of the 84 (69%) patients; of the remaining 26 patients, 15 had a positive family history, 4 had related parents, 4 had incomplete data, and 3 fulfilled less than 5/8 criteria.

In particular, among those for whom the information was available, fever was present in 89% of patients, splenomegaly in 96%, bi-cytopenia in 88%, hypertriglyceridemia and/or hypofibrinogenemia in 95%, haemophagocytosis in 78%, hyperferritinemia in 77%, and low or absent NK cell activity in 98% (table 1).

The combination of fever, splenomegaly and thrombocytopenia was present in 65 of 77 patients (84%) evaluable for these three parameters; of them, 55 had information on ferritin level, which turned to be elevated in 39 (71%); otherwise, 59 of the 78 had

information on fibrinogen level, which turned to be reduced in 33 (56%); finally, all of the 78 had information on hemophagocytosis, which turned to be present in 54 (69%) (Figure 1).

CNS involvement was reported in 60% of the patients: 30/80 (37%) had neurologic 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 9 splice errors (Fig. 2).

A total of 23 different mutations were observed at the homozygous state in 42 individuals from 36 unrelated families; their presenting features are summarized 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%), 9 Caucasian, one Asian and one Hispanic. Nine homozygous patients had a median age at the diagnosis of 3 months (range, 2 to 12); 4 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 8 individuals from 6 families of Caucasian origin. Four patients carried the mutation at the homozygous state: age at diagnosis was 99 and 139 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 all the three patients tested.

The p.**R928C** missense mutation **(c.2782C>T)** was identified in 8 patients from 7 unrelated families, 6 Caucasian and one Hispanic. This mutation falls in the C2B domain; its predicted impact is controversial, i.e. 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 has 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 4 unrelated patients (3 Caucasian, 1 origin not reported). All were compound heterozygous with different frameshift (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 male (UPN 397) presenting with typical FHL, reduced CD107 expression and NK-activity. 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 them 17 had quantitative evaluation, analysing activated NK cells upon co-culture with K562, with a mean value of 14.5% of $\Delta CD107a^{+}$ 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 them 17 had quantitative evaluation of the lytic units (LU), analyzing 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 them, 11 within the functional domains of the protein, while the remaining 8 fall 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 compared to 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 ($p<0.0001$) (Fig. 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 (61%) evaluable patients in group 1 versus 27 of 33 (81%) 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 versus 39 of 43 (90%) 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 them 17 had quantitative evaluation, analyzing activated NK cells upon co-culture with K562, with a mean value of 14.5% of $\Delta CD107a^+$ cells (SD 11.7, SE 2.8). This value was significantly inferior to that of healthy controls, both adults and infants ($p<0.0001$). Analysing separately patients of group 3 from those of groups 1 and 2, they did not differ from each other and both were significantly lower than healthy controls (Figure 4, panel A).

Reduced or absent NK cytolytic activity was found in 44 of the 45 patients analysed (98%). Of them 17 had quantitative evaluation of the lytic units (LU), analyzing 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 ($p<0.005$). Mean LU value was not significantly lower in patients from group 3 than those in groups 1 and 2 (Figure 4, panel B).

Comparison between 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 worldwide, the predominance of Caucasian patients in this series only reflects the reporting bias of the European location of the contributing centres. However, due to increasing immigrations, 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 what previously observed 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 like being fully 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 tumor cell lines or EBV-infected cells.[30] However, FHL3 NK cells, also tested upon activation, displayed a significantly lower degranulation capacity and cytotoxic activity as compared to healthy controls (Fig. 4), while for other diseases (e.g. FHL4, FHL5 and GS2) the defects can be prevalently detected using resting NK cells.[25,26] This allows to perform functional assays 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 result in absence of the Munc13-4 protein.[31] Both ultimately developed FHL3, although at a different age, i.e. 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-modifier genes are involved, we are not able to document, at present. All the above should be taken into account when counseling asymptomatic family members with documented genetic defect.

The diagnostic criteria for HLH have been originally defined by the Histiocyte Society in 1994, and then revised in 2004.[10,11] In the present series, the combination of 5 of the 8 items, i.e. 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 have 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 “leukemia”, demonstrates

hemophagocytosis, the diagnosis is even more supported. In this series, the combination of these 4 findings was present in 69% of the patients evaluated also 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 the suspicion of FHL3; when associated with evidence of increased ferritin level, these features may be considered as a very sensitive tool to address the diagnostic work-up already 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 central nervous system, 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 neurologic 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 FHL2 patients showing CNS involvement.[28,37]

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.[38] 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 yet did not include any Japanese or Korean patient.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 in 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 aberrations.

Overall, one third of the mutations were missense, including 7 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 proven 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 even despite specific treatment. Although mutation analysis remains the gold standard for diagnosis of FHL, results are not readily available. We have found defective degranulation in almost all of the evaluated FHL3 patients. 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 flowcytometry[30,41,42] at national reference laboratories. All patients fulfilling the diagnostic pattern and with degranulation defect should be considered eligible for immediate empiric treatment, followed by directed mutation analysis to support indication to HSCT as a curative

therapeutic program. Given the high frequency of mutations leading to splicing errors, the strategy of analysis must include the RNA study.

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Table 1. Presenting features in 84 patients with familial hemophagocytic lymphohistiocytosis and *UNC13D* mutations

	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)
Hypofibrinogenemia	37/60 (62)
Hypertriglyceridemia	49/63 (78)
Hyperferritinemia	43/56 (77)
Hemophagocytosis	63/81 (78)
Defective NK activity	44/45 (98)

Table 2 . Main clinical features and initial laboratory evaluation of 42 patients with familial hemophagocytic lymphohistiocytosis type 3 and homozygous mutations

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

NA: Not Available. UPN: unique patient number; Novel mutations are in bold. ** Siblings

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

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

[b] In the cytotoxicity assays, NK activity was considered "absent" if below 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.

[c] Munc13-4 was tested by western blot.

Table 3. Main clinical features and initial laboratory evaluation of 11 patients with familial hemophagocytic lymphohistiocytosis with compound heterozygous novel mutations

Allele 1 Mutation	Allele 2 Mutation	UPN	Ethnic group	Age at diagnosis (months)	Fever	Splenomegaly	CNS disease	Anemia	Neutropenia	Thrombocytopenia	Hyperferritinemia	Hypertriglicerideemia	Hypofibrinogenemia	CD107 expression[a]	NK activity[b]	MUNC 13-4 expression[c]
c.403insC (p.Y135Fs)	c.627delT (p.T209Fs)	481	Caucasian	28	+	+	+	+	+	+	+	+	-	Reduced	Reduced	NA
c.419T>C (p.I140T)	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.2437-2439delAACinsT (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	Reduced	Absent	Absent

NA: Not Available. Novel mutations are in bold.
* This patient also carried the c.2782C>T (p.R928C) mutation.
[a] In the degranulation assays, CD107 expression was considered “absent” if below 5% or 10% in resting or activated NK cells, respectively; “reduced” if significantly ($p<0.05$) lower than controls.
[b] In the cytotoxicity assays, NK activity was considered “absent” if below 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..
[c] Munc13-4 was tested by western blot.

LEGEND TO FIGURES

Figure 1. Distribution of the frequency of some diagnostic parameters for HLH and pattern of their association.

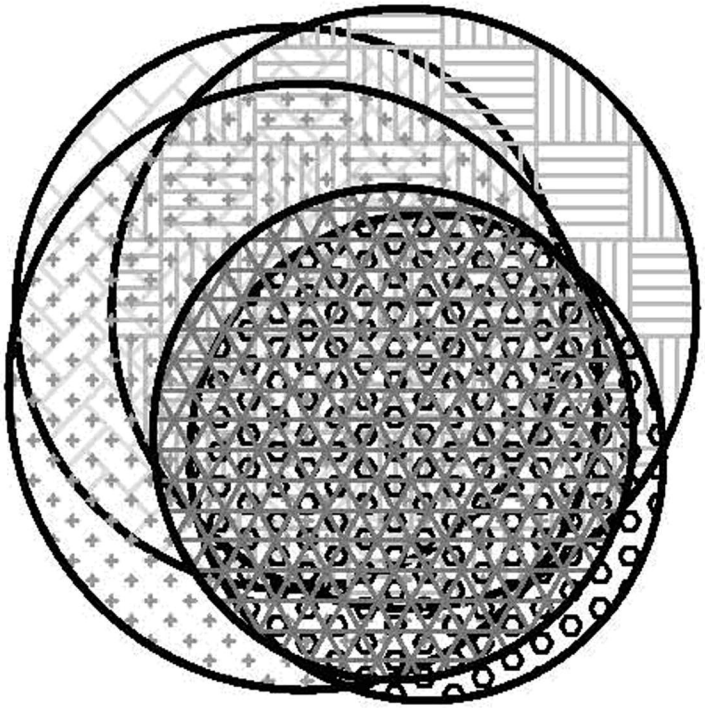
Figure 2. Details of 54 different *UNC13D* mutations observed in 84 patients with FHL3 from 69 families. Mutations are spread over the entire gene. Disruptive mutations are indicated in bold.

Figure 3. Age at onset of 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.

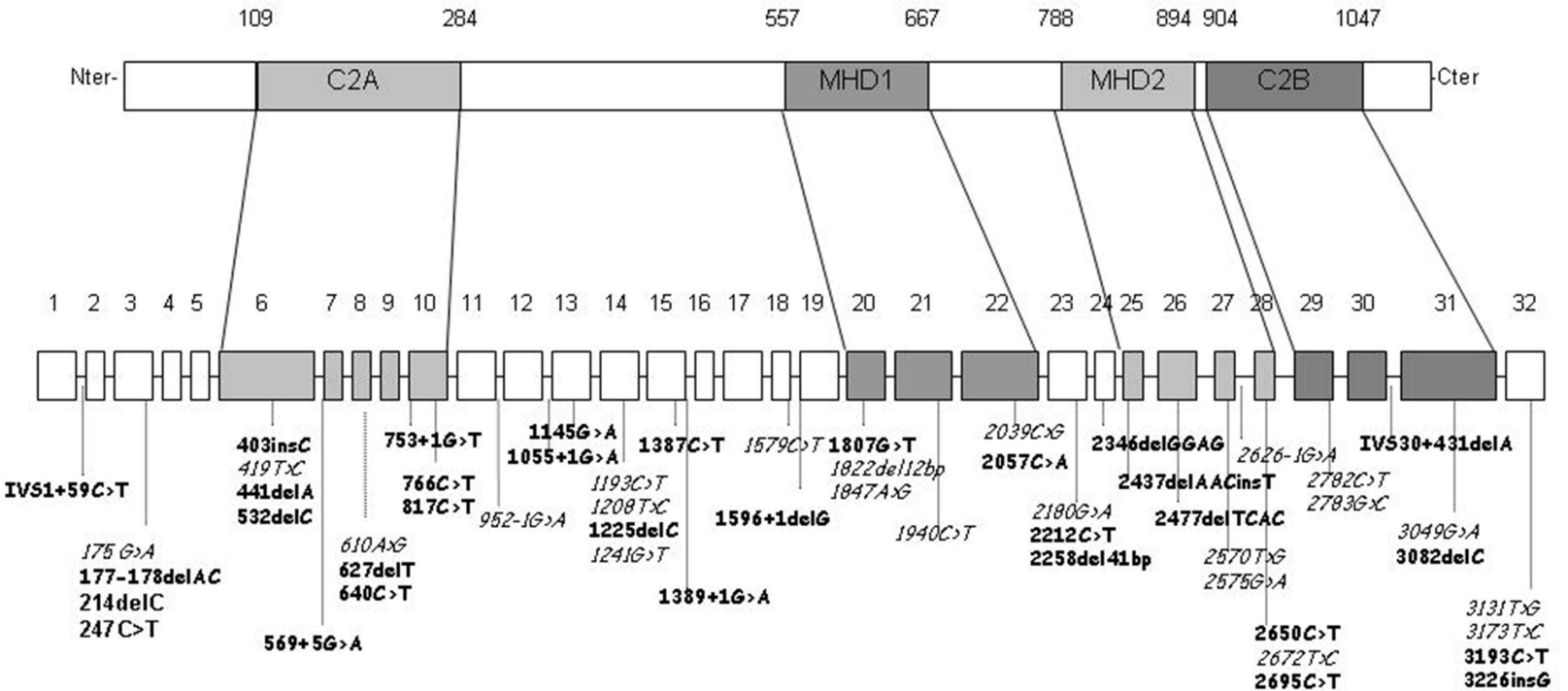
Figure 4. Impaired degranulation and cytotoxicity by activated NK cells from FHL3 patients. Polyclonal activated NK cell populations derived from healthy adult and infant donors, and from FHL3 patients, considered separately as group 1+2 and group 3, were analysed for surface expression of CD107a after stimulation with K562 (data are expressed as % 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; n.s., not significant.

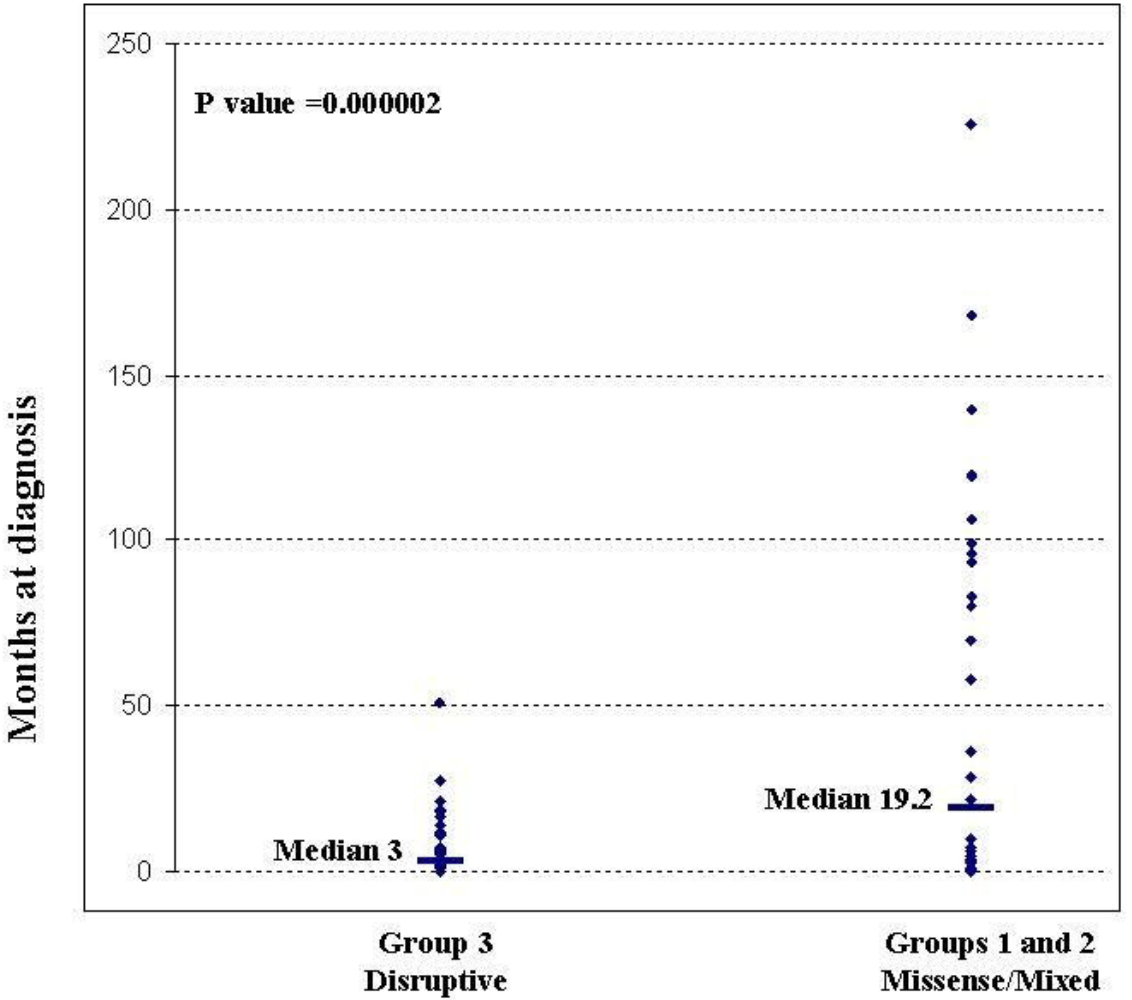
Figure 5. Age at onset of FHL in patients with two disruptive mutations is significantly younger in patients with FHL2 than in patients with FHL3. Each point represents one person, lines indicate mean values.

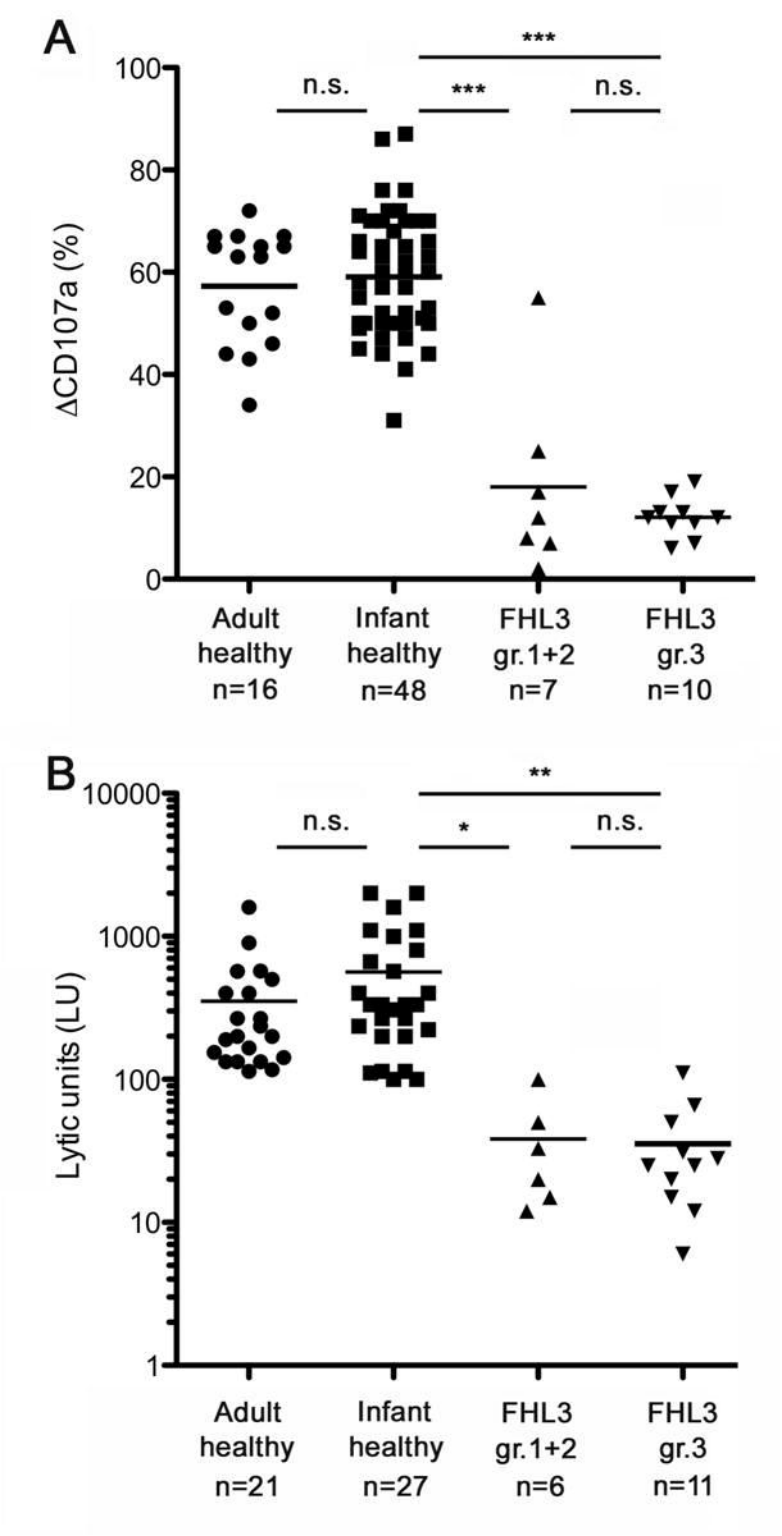
Figure 6. Comparison of age at onset in 14 families with FHL3 and at least two affected siblings.

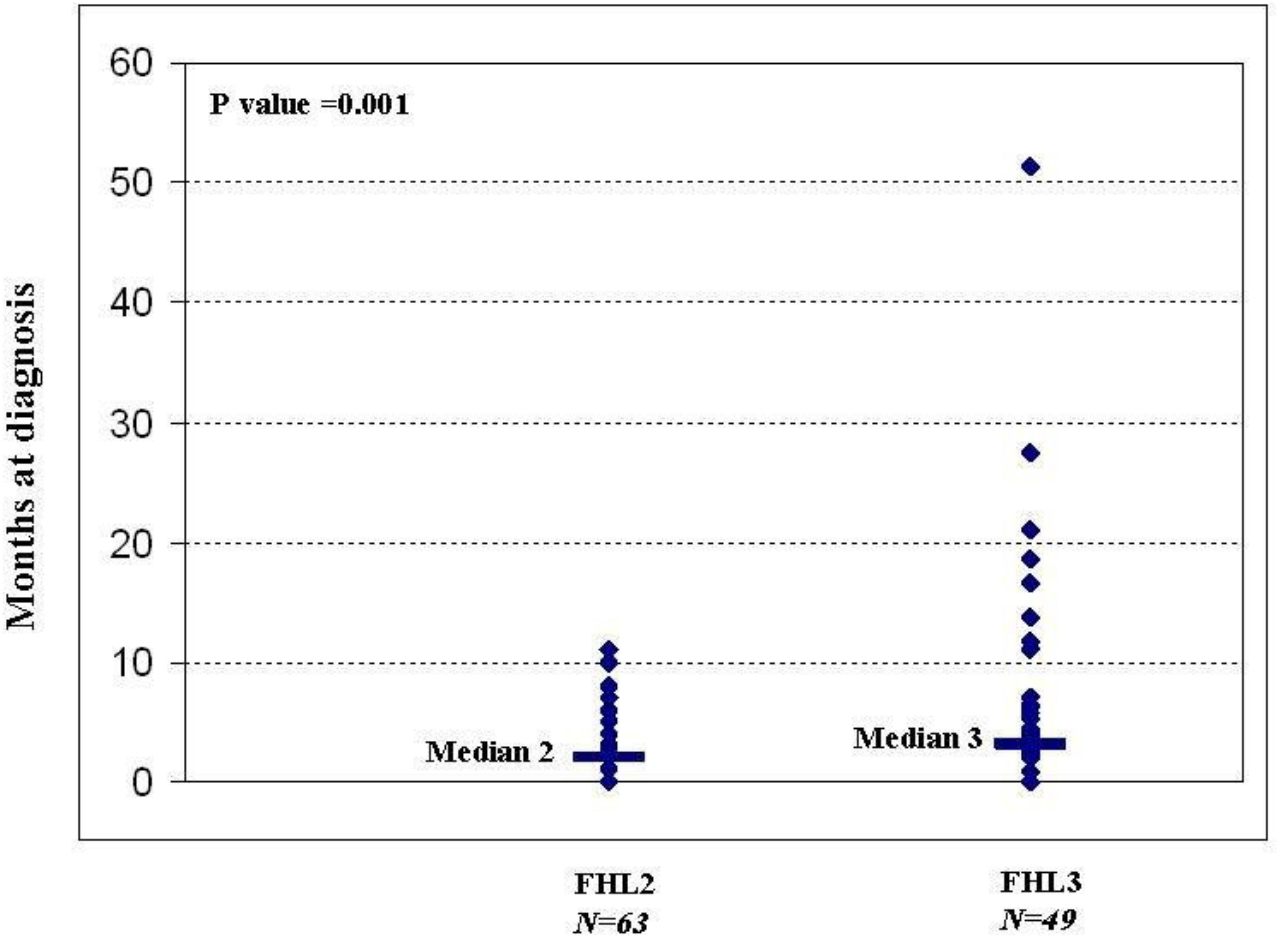


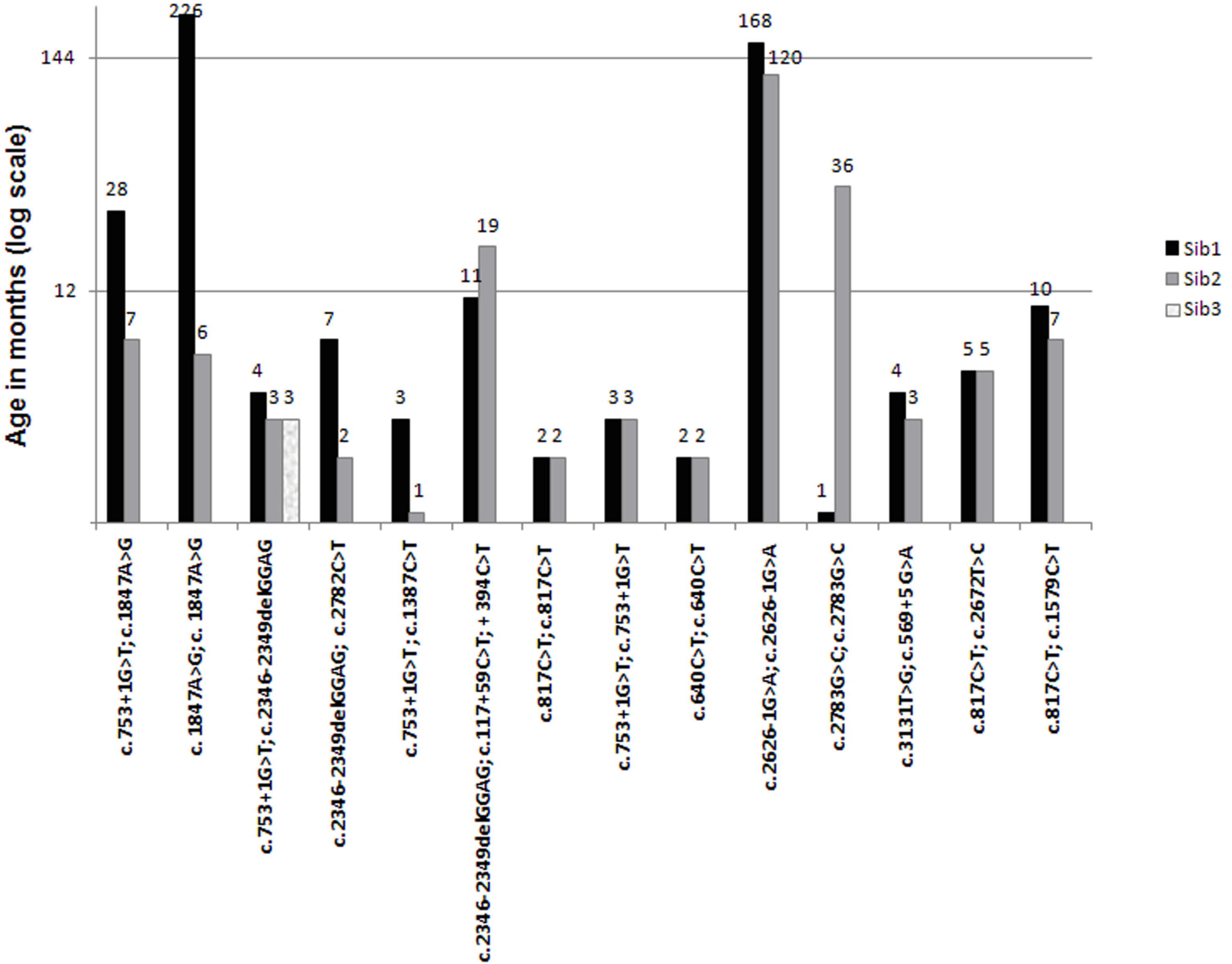
Fever	89%
Splenomegaly	96%
Thrombocytopenia	94%
Haemophagocytosis	78%
Hyperferritinaemia	77%
Fever + Splenomegaly	88%
Fever + Splenomegaly+ Thrombocytopenia	84%
Fever + Splenomegaly+ Thrombocytopenia+ Hyperferritinaemia	71%
Fever + Splenomegaly+ Thrombocytopenia+ Haemophagocytosis	69%
Fever + Splenomegaly+ Thrombocytopenia+ Haemophagocytosis+ Hyperferritinaemia	57%


109
284
557
667
788
894
904
1047
Nter-
C2A
MHD1
MHD2
C2B
-Cter
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32
IVS1+59C>T
175 G>A
177-178delAC
214delC
247 C>T
403insC
419TxC
441delA
532delC
569+5G>A
610AxG
627delT
640C>T
753+1G>T
766C>T
817C>T
952-1G>A
1145G>A
1055+1G>A
1193C>T
1208TxC
1225delC
1241G>T
1387C>T
1389+1G>A
1579C>T
1596+1delG
1807G>T
1822del12bp
1847AxG
1940C>T
2039CxG
2057C>A
2180G>A
2212C>T
2258del41bp
2346delGGAG
2437delAACinsT
2477delTCAC
2570TxG
2575G>A
2626-1G>A
2650C>T
2672TxC
2695C>T
2782C>T
2783GxC
IVS30+431delA
3049G>A
3082delC
3131TxG
3173TxC
3193C>T
3226insG


P value =0.000002
Months at diagnosis
250
200
150
100
50
0
Median 3
Median 19.2
Group 3
Disruptive
Groups 1 and 2
Missense/Mixed


A
***
n.s.
***
n.s.
100
80
60
40
20
0
ΔCD107a (%)
Adult healthy n=16
Infant healthy n=48
FHL3 gr.1+2 n=7
FHL3 gr.3 n=10
B
**
n.s.
*
n.s.
10000
1000
100
10
1
Lytic units (LU)
Adult healthy n=21
Infant healthy n=27
FHL3 gr.1+2 n=6
FHL3 gr.3 n=11


P value =0.001
Months at diagnosis
60
50
40
30
20
10
0
Median 2
Median 3
FHL2
N=63
FHL3
N=49


Age in months (log scale)
144
12
226
168
120
36
28
7
19
11
7
6
4
3 3
7
2
3
1
2 2
3 3
2 2
1
4
3
5 5
10
7
Sib1
Sib2
Sib3
c.753+1G>T; c.1847A>G
c. 1847A>G; c. 1847A>G
c.753+1G>T; c.2346-2349deKGGAG
c.2346-2349deKGGAG; c.2782C>T
c.753+1G>T ; c.1387C>T
c.2346-2349deKGGAG; c.117+59C>T; + 394C>T
c.817C>T; c.817C>T
c.753+1G>T; c.753+1G>T
c.640C>T; c.640C>T
c.2626-1G>A; c.2626-1G>A
c.2783G>C; c.2783G>C
c.3131T>G; c.569+5G>A
c.817C>T; c.2672T>C
c.817C>T; c.1579C>T