Genetic predisposition to hemophagocytic lymphohistiocytosis: Report on 500 patients from the Italian registry

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Background: Hemophagocytic lymphohistiocytosis (HLH) is a rare life-threatening disease affecting mostly children but also adults and characterized by hyperinflammatory features.
A subset of patients, referred to as having familial hemophagocytic lymphohistiocytosis (FHL), have various underlying genetic abnormalities, the frequencies of which have not been systematically determined previously.
Objective: This work aims to further our understanding of the pathogenic bases of this rare condition based on an analysis of our 25 years of experience.
Methods: From our registry, we have analyzed a total of 500 unselected patients with HLH.
Results: Biallelic pathogenic mutations defining FHL were found in 171 (34%) patients; the proportion of FHL was much higher (64%) in patients given a diagnosis during the first year of life. Taken together, mutations of the genes *PRF1* (FHL2) and *UNC13D* (FHL3) accounted for 70% of cases of FHL. Overall, a genetic diagnosis was possible in more than 90% of our patients with FHL. Perforin expression and the extent of degranulation have been more useful for diagnosing FHL than hemophagocytosis and the cytotoxicity assay. Of 281 (56%) patients classified as having "sporadic" HLH, 43 had monoallelic mutations in one of the FHL-defining genes. Given this gene dosage effect, FHL is not strictly recessive.
Conclusion: We suggest that the clinical syndrome HLH generally results from the combined effects of an exogenous trigger and genetic predisposition. Within this combination, different weights of exogenous and genetic factors account for the wide disease spectrum that ranges from HLH secondary to severe infection to FHL. (J Allergy Clin Immunol 2016;137:188-96.)

Key words: *Hemophagocytic lymphohistiocytosis, PRF1, UNC13D, immunologic tests*

In an article published in *The Lancet* in 1939, Bodley-Scott and Robb-Smith reported 4 patients originally given a diagnosis of "atypical Hodgkin disease."[1] The patients had unrelenting fever, lymphadenopathy, and rather massive hepatosplenomegaly. The authors stated that "where the cause of a morbid process is unknown its diagnosis commonly rests on the changes in the patient's tissues; thus ultimately the diagnosis can only be made with certainty by the microscope." In their cases the ultimate microscopic analysis was made postmortem because all 4 patients died. The authors were struck by the massive abundance of histiocytes in the spleen, liver, and other organs and by the prominent feature of erythrophagocytosis: they found 6 similar patients in the literature and coined the term "histiocytic medullary reticulosis."[1] Only the initial letter of histiocytes has survived in current acronyms, but we have little doubt that that article was the first clinicopathologic description of what we call today hemophagocytic lymphohistiocytosis (HLH). Some years later, Farquhar and Claireaux[2] described the first example of familial hemophagocytic lymphohistiocytosis (FHL), an entity for which other names in the literature have been "genetic" or "primary" HLH.[3,4] FHL is now defined on the basis of biallelic mutation in one of a set of functionally related genes or on familial recurrence. For clinical purposes, disease reactivation after initial remission has also been regarded as supporting the diagnosis of FHL.

From the functional point of view, in patients with FHL, defects of killing by T cells and natural killer (NK) cells have been prominent, and these can be identified by using appropriate tests. Over the last 15 years, it has been discovered that these defects are the consequence of mutations in one of a few genes (*PRF1*, *UNC13D*, *STX11*, and *STXBP2*) that are involved in exocytosis

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Abbreviations used
FHL: Familial hemophagocytic lymphohistiocytosis
HLH: Hemophagocytic lymphohistiocytosis
HSCT: Hematopoietic stem cell transplantation
MAS: Macrophage activation syndrome
NK: Natural killer
XLP: X-linked lymphoproliferative syndrome

of cytoplasmic granules and hence in perforin-mediated killing of target cells.[5-9] FHL can also be caused by mutations in other genes (*SH2D1A*, *XIAP*, *RAB27A*, *LYST*, and *AP3B1*) associated with the X-linked lymphoproliferative syndrome (XLP) disorders XLP1 and XLP2,[10] Griscelli syndrome type 2,[11] Chediak-Higashi syndrome,[12,13] and Hermansky-Pudlak syndrome.[14] The genetic defects in these patients (who sometimes have additional readily recognizable features) make them unable to effectively cope with the challenging pathogen, thus giving the picture of FHL.[15]

Cases with no evidence of familial recurrence have been called "secondary," but we prefer to call them "sporadic." For instance, HLH can develop as a complication of juvenile idiopathic arthritis (these cases have been called macrophage activation syndrome [MAS]) or during immunosuppressive therapies for cancer or autoimmune disorders,[4,16] although it can occur also in the course of protozoal (leishmaniasis and malaria), rickettsial,[17] or mycobacterial infection. In a proportion of cases of sporadic HLH, one finds cytocidal defects similar (although less profound) to those seen in patients with FHL. In the remaining cases of sporadic HLH, tests currently in use do not detect functional abnormalities, although we suspect that they exist.

The natural course of HLH is life-threatening (almost invariably so in patients with FHL) unless appropriate therapy is promptly instituted.[3] Two trials of the Histiocyte Society have established the combination of etoposide and dexamethasone as the standard of care.[18,19] This treatment is aimed to bridging the time gap until it is practical to carry out an allogeneic hematopoietic stem cell transplantation (HSCT), which, by replacing genetically defective bone marrow–derived cells with normal cells, provides cure for most patients.[20-22] Patients with sporadic disease have a similar clinical picture, and they respond to the etoposide/dexamethasone protocol as in patients with FHL. However, unlike patients with FHL, once in remission, they remain disease free, without any further therapy.[3,4,18,19] Thus patients with HLH are a dual diagnostic challenge. First, the clinical suspicion of HLH ought to be confirmed quickly, so that treatment can be started promptly to prevent organ damage and the risk of a fatal outcome. Second, it is important to distinguish FHL from sporadic HLH, so that HSCT can be organized speedily for patients with FHL and, just as importantly, patients with sporadic HLH are spared unnecessary HSCT.

Over the past 25 years, we have collected data on patients in whom a clinical diagnosis of HLH has been made by the attending clinicians. Here we report on those 500 patients in whom the diagnosis of HLH was subsequently confirmed.

METHODS

Starting from 1989, we centralized patient information and biologic samples to support the provisional clinical diagnosis of HLH and to perform immunologic and genetic studies of Italian patients, who were defined as such if living in Italy.[3] All centers of the national pediatric hematology-oncology cooperative group *(Associazione Italiana Ematologia Oncologia Pediatrica)* agreed to refer their patients' samples and information. Furthermore, in some cases samples of adult patients with a similar clinical picture are also referred.

HLH was defined according to the diagnostic criteria recommended by the Histiocyte Society (see Table E1 in this article's Online Repository at www.jacionline.org).[23] In 98 patients preliminarily reported as having HLH, the diagnosis was modified (acute infection, n = 19; cancer [lymphoma, n = 5; leukemia or myelodysplastic syndrome, n = 8; or brain tumor, n = 2], n = 15; liver failure, n = 12; encephalopathy, n = 12; autoimmune lymphoproliferative syndrome, n = 8; cytopenia, n = 8; other congenital disorder, n = 7; lysinuric protein intolerance, n = 5; primary immune deficiency, n = 4; congenital storage disease, n = 4; and Langerhans cell histiocytosis, inflammatory bowel disease, healthy subject, and other undefined, n = 1 each). Therefore these patients were excluded from this analysis.

Data on the family history and clinical and laboratory presenting features are collected on a specific form and stored in a dedicated Microsoft Access database. A unique patient number encodes the patients.

Peripheral blood samples from the patients are shipped overnight. Patients are tested before treatment start or during steroid monotherapy, which does not modify the results.[24] We perform immunologic screening assays,[25-30] and in case of abnormalities compatible with the clinical suspect, mutation analysis is started. In some patients receiving a diagnosis before immunologic assays were available or material from whom was inadequate for functional assays, mutation analysis was directly performed. The strategy of mutation analysis is as follows: in patients with defective perforin expression, *PRF1* is sequenced; in patients with a degranulation defect, *UNC13D*, *STX11*, and *STXBP2* are sequenced in that order; in patients with pigment deficiency, *RAB27a* and then *LYST* are sequenced; in male patients with defective SAP expression and/or inhibitory instead of activating 2B4 receptor function, *SH2D1A* is sequenced; and in male patients XIAP staining is also performed, and *XIAP* is sequenced. Archive cases with suspected but undefined genetic disease were progressively retrieved and analyzed when additional genes became available for testing.

Immunologic analyses and mutation analysis

Measurement of protein expression, granule release assays, cytotoxicity assays, and mutation analysis were performed, as previously described.[25-30] Details are available in the Methods section in this article's Online Repository at www.jacionline.org.

Statistical analysis

Statistical significance of the differences between the presence or absence of biallelic mutation for each age group has been evaluated by using the χ^2 test (GraphPad Prism 6; GraphPad Software, La Jolla, Calif). Differences have been considered significant at a P value of less than .05.

Ethics statement

The attending physician obtained written informed consent for data collection, immunologic studies, and genetic studies for all patients and family members. The study was approved by the Institutional Review Board of the A.O.U. Meyer, Florence, Italy. All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki.

RESULTS

Study population

The mean annual accrual to the Italian Registry increased steadily over the study period (see Table E2 in this article's Online Repository at www.jacionline.org), with a total of 500 patients enrolled. There were 259 male and 241 female patients. Age at the time of diagnosis ranged from 0 to 60 years (Fig 1), with a median of 2.2 years. Although the vast majority of patients were children, 44 (8.8%) were older than 18 years at the time of diagnosis.

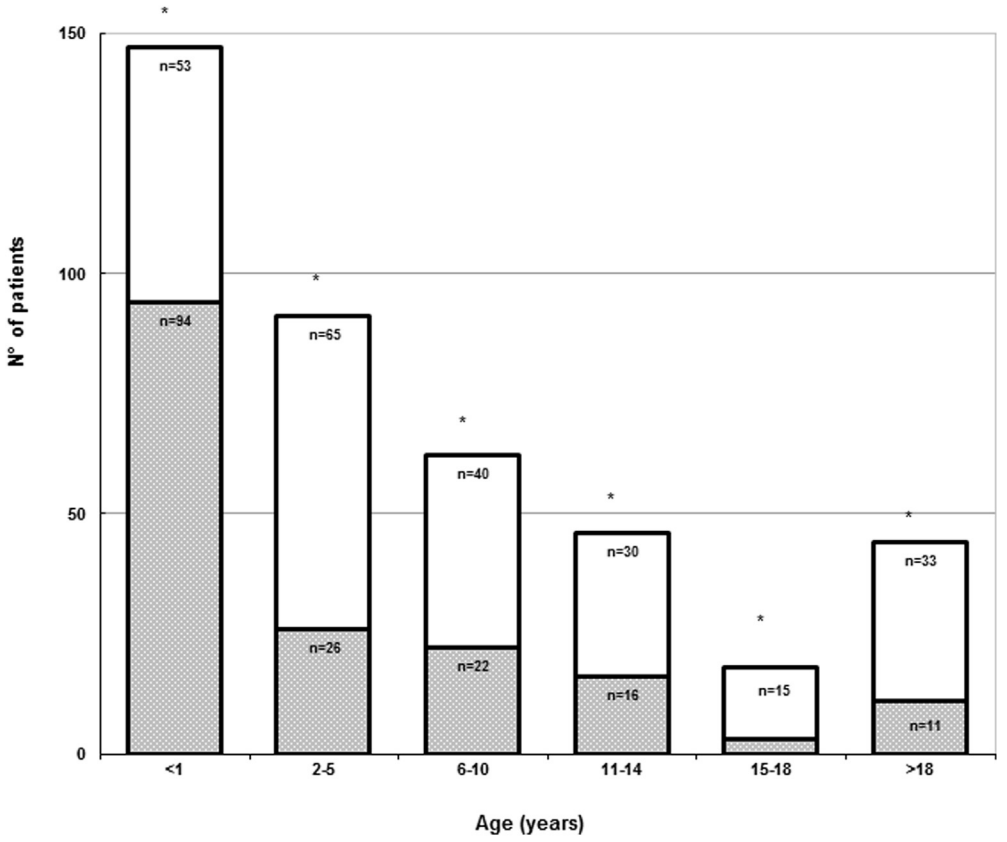

FIG 1. Distribution of patients with FHL (biallelic mutations; *gray bars*) or sporadic HLH (no biallelic mutations; *white bars*), according to age at diagnosis. The number of patients for each age group is indicated within each bar. $*P < .001$, χ^2 test.

The origin of the families was as follows: southern European, 433 (87%); eastern European, 14; African, 12; Asian, 13; Hispanic, 6; other/not known, 22. Parental consanguinity was reported in 40 (8%) and familial recurrence of the disease in 75 (15%) patients.

Immunologic studies

In 33 (6.6%) cases the blood sample received was insufficient or inadequate for any diagnostic study. In a further 95 cases insufficient cell viability prevented us from performing functional assays. In the remaining 372 (74%) cases we performed at least 1 immunologic test (Fig 2 and Table I). Absent perforin expression was always predictive of biallelic *PRF1* mutations. A complete degranulation defect was also predictive for biallelic mutations in one degranulation-related gene (*UNC13D*, *STXBP2*, *RAB27A*, or *LYST*). Seven of 8 patients with FHL with normal perforin and degranulation assay results fell within the XLP category, confirming that the degranulation assay is not the test of choice for these patients.

Mutation analysis

DNA studies were carried out in 426 (85%) patients, of whom (1) 275 had abnormal results on at least 1 immunologic assay; (2) 95 could not have immunologic screening performed; and (3) 56, despite normal immunologic test results, were suspected of having FHL based on 1 of the following: consanguinity, familial

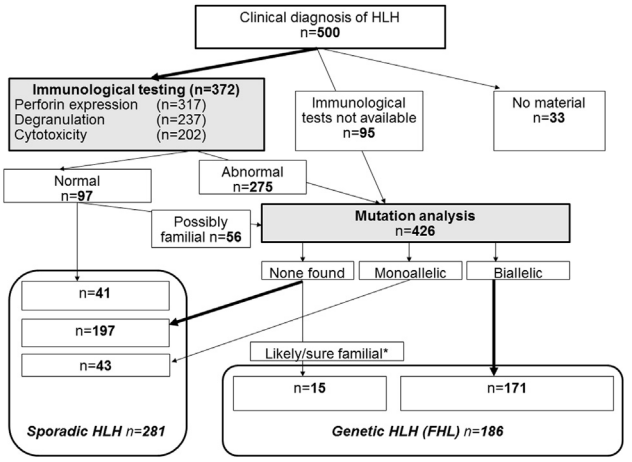

FIG 2. Diagnosis and classification of HLH. Distribution of 500 patients with HLH according to the presence or absence of biallelic mutations in any of the FHL-related genes is shown. Not all patients were investigated for the 3 functional assays. In 275 patients functional testing was followed by mutation analysis. Patients were classified as having sporadic HLH if they had no evidence of a severe functional defect and did not have biallelic mutations in any FHL-related genes. Patients were classified as having genetic HLH (or FHL) if they had biallelic mutations, with the only exception of 15 patients given a diagnosis of FHL for clinical reasons in whom mutations were not found. A possible simple explanation is that the underlying gene or genes have yet to be discovered. Their details are summarized in Table E3.

TABLE I. Correlation of perforin expression and degranulation assays in 372 patients with HLH in whom at least 1 of the immunologic assays could be performed

Perforin expression	Degranulation				
	Normal	Reduced	Complete defect	Not performed	Total
Normal	106 (8)	34 (8)	**29 (29)**	46 (13)	215 (58)
Reduced	26 (5)	10 (1)	**3 (3)**	33 (8)	72 (17)
Absent	**12 (12)**	**1 (1)**	0	**17 (17)**	**30 (30)**
Not performed	11 (2)	2 (0)	**1 (1)**	41* (25)	55 (28)
Total	155 (27)	47 (10)	**33 (33)**	137 (63)	372 (133)

The number of patients with biallelic mutations defining the diagnosis of FHL are shown in parentheses. All of the patients with absent perforin expression or complete degranulation defects (shown in boldface) had biallelic mutations in *PRF1* or one degranulation-related gene, respectively.
*In these 41 patients (32 of whom received a diagnosis within the year 2000 and none during the last 5 years), the only immunologic test performed was the cytotoxicity assay, results of which were normal in 5, reduced in 10, severely depleted in 26 patients.

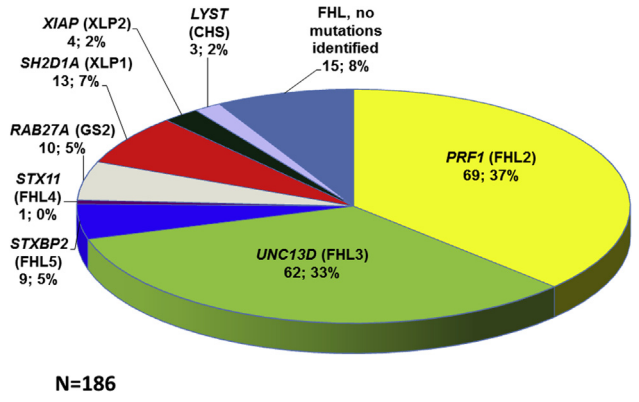

FIG 3. Breakdown of the different genetic subtypes in 171 patients with FHL or FHL-related disease. For each subtype, the name of the gene, the abbreviation of the disease subtype, the absolute number, and the percentage are shown. Furthermore, we include as FHL one subgroup of 15 patients with either familial recurrence and/or refractory/recurrent disease despite specific therapy and/or repeatedly documented severe functional defect in degranulation or cytotoxicity assays (see Table E3).

recurrence, pigment deficiency, or, in a minority of patients, disease reactivation.

Patients with FHL

Mutation analysis revealed biallelic pathogenic mutations in 171 (40%) patients. According to the genes in which we found mutations, these were further subclassified into genetic subtypes (Fig 3).

In the 69 patients with FHL2, we identified 34 different *PRF1* mutations (Table II); 28 patients were homozygous for their *PRF1* mutation. The most frequent mutations were c.1122G>A (p.W374X), which is present in 19 patients; c.272C>T (p.A91V), which is present in 11 patients; c.657C>A (p.Y219X), which is present in 10 patients; and c.695G>A (p.R232H), which is present in 9 patients. The median age at diagnosis was 0.2 years (0.18 years for homozygotes and 0.29 years for biallelic heterozygotes), and the range was 0 to 49 years.

In the 62 patients with FHL3, we found 37 different *UNC13D* mutations (Table II). The most frequent mutations were c.753+1G>T (splice error), which was present in 19 patients; c.2346_2349delGGAG (p.R782Sfs), which was present in 11 patients; and c.1847A>G (p.E616G), which was present in 8 patients. Twenty-two patients were homozygotes, and 32 had at least 1 splice defect mutation. Just like in the FHL2 group, the median age at diagnosis was earlier in homozygotes (0.32 years) than in heterozygotes (1.5 years).

TABLE II. Different mutations identified in patients with FHL

	PRF	*UNC13D*	*STXBP2*	*STX11*	*RAB27A*
Missense	20	11	4	0	1
Nonsense	6	8	0	0	1
Deletions/insertions	8	12	3	1	3
Splicing	0	6*	0	0	0
Total	34	37	7	1	5

*Transcript defects were all documented at the RNA level.

In 26 families we had 2 sibs in our registry. In 16 cases the age at which the disease manifested was very close in the 2 affected sibs. However, in 9 cases we observed an age difference of between 46 and 207 months, and in 1 case one sib had the disease at 6.7 years, whereas the other remains unaffected at the age of 25 years (Fig 4).

In 15 patients (3.5% of the patients in whom we performed mutation analysis and 3% of the total patients enrolled, see Table E3 in this article's Online Repository at www.jacionline.org), we found no pathogenic mutations in FHL-related genes in spite of the fact that we had strongly suspected FHL on the grounds of familial recurrence (n = 6), confirmed severe immunologic defect (n = 3), immunologic defect associated with pigment deficiency (n = 1), or recurrent and fatal disease (n = 5). These last 5 patients were given diagnoses when 24 months old or less, between 1983 and 1996; of them, 1 patient had absent NK cell activity, and mutation analysis could not be completed in any of them because lack of material; if this had been done, we hypothesized that they might have likely been classified in one of the known FHL subtypes.

Patients with sporadic HLH

Two hundred eighty-one patients (56% of the total) were categorized as having sporadic HLH on the following grounds. First, in 41 patients immunologic test results were normal. In these patients mutation analysis was not carried out. Second, in 197 patients we found no mutations in FHL-related genes. Third, 43 patients had monoallelic mutations in 1 (n = 41) or 2 (n = 2) FHL-related genes (*PRF1*, n = 25; *UNC13D*, n = 10; *STX11*, n = 2; *STXBP2*, n = 6; and *RAB27A*, n = 2; Fig 2).

None of the patients with sporadic HLH had a complete defect of either perforin expression or degranulation. To establish correlations between the outcome of immunologic assays and monoallelic mutations in patients with sporadic HLH, we found that of 55 patients with reduced (but not absent) perforin expression, 47 were sequenced, and 15 (32%) had monoallelic *PRF1* mutation. Of 37 patients with reduced (but not absent) degranulation, 35 were sequenced, and 8 (23%) had monoallelic mutation in *UNC13D* (n = 4), *STXBP2* (n = 3), or *RAB27A* (n = 1).

Survival

Overall, at the time of writing, 159 (31.8%) of the 500 patients had died. In the various subgroups the mortality rates were as follows: 50.8% of the 171 patients with FHL with biallelic

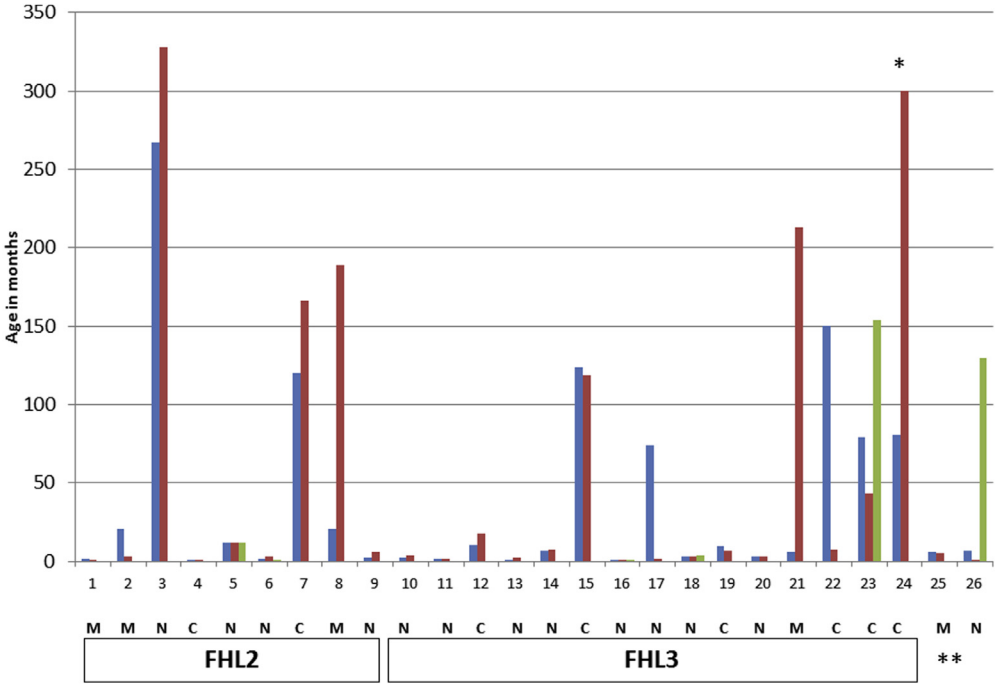

FIG 4. Intrafamilial variation of age at onset of FHL in 26 families with more than 1 affected case. In families 23 and 26, 3 affected cases are reported. In families 4, 9, and 18, the siblings were concordant twins. *This sibling of patient 24 is now 25 years old and has not yet had the disease. The FHL genetic subtype is indicated at the bottom, with the exception of family 25 (FHL5) and family 26 (Griscelli syndrome type 2). The type of mutations are abbreviated as follows: *C*, combination of 1 nonsense and 1 missense mutation; *M*, biallelic missense mutations; *N*, biallelic nonsense mutations. **Case 25, FHL5; case 26, Griscelli syndrome type 2.

mutations; 73% of the 15 patients with FHL with no evidence of mutations; and 41.6% of the 72 patients not analyzed for mutations.

Among the 281 sporadic cases, 11% died (including 2 after HSCT) at a median of 29 days from diagnosis; there was no significant difference in mortality between patients with monoallelic mutations (5/43 [12%]) and those with no mutations (26/197 [13%]; $P = .84$, χ^2 test; odds ratio, 0.91).

DISCUSSION

In this article we summarize the experience accumulated over 25 years through the study of 500 unselected patients with HLH resident in Italy. Among 50 units that normally refer patients to our registry, 14 pediatric hematology-oncology units have referred more than 10 cases each (range, 11-48 cases). The steady increase in patient accrual over the study period must mean that there is increased awareness of HLH among pediatricians and other doctors. Overall, about one third of the patients had biallelic mutations (ie, they had FHL by definition). Thus the frequency of patients with FHL in Italy is comparable with that reported in Nordic countries (around 1:50,000 children).[31] Interestingly, over the years, the ratio between FHL cases and sporadic HLH cases has gradually decreased by more than 3-fold (see Table E2), indicating increased recognition not just of FHL but also of HLH in general.

The median age at diagnosis was 2.2 years. Nearly two thirds (94/147 [64%]) of patients given a diagnosis before the age of 1 year had FHL; this proportion decreased to 27% in the second year and remained roughly the same at higher ages (Fig 1), including in adults. This still relatively high proportion of FHL in adults has major implications for adult hematology.[32-34] Another surprising finding was that when more than 1 sib was affected within the same sibship, in two thirds of cases, there was a wide gap between the age at diagnosis of the 2 (or 3) sibs. In an extreme case a child had the disease at 6.7 years, whereas his sister, who also has biallelic mutations, has not yet had HLH by the age of 25 years. As a kind of internal control, in one third of cases, the age at which 2 sibs had HLH was very similar (Fig 4).

Increasing immigration from countries with limited resources brings new citizens into Italy. Among 55 such patients, 18 harbored biallelic mutations, and their subtype distribution appears no different from that of the autochthonous patients. Our registry also contains more than 500 additional cases who live in other countries: we have not included them in our analysis because in these referrals from abroad, there might have been considerable selection bias.

Identifying FHL

A priority of a reference laboratory must be to differentiate FHL from sporadic HLH in as many cases as possible as promptly as possible. One major finding of our study is that we did not find biallelic mutations in only 8% of patients (3.5% of the total) presumed to have FHL. The most likely explanation is that this small minority of patients with FHL might have mutations in 1 or more gene(s) that have not yet been identified as FHL related. At any rate, in the large majority of cases, we are able to classify correctly patients as having FLH versus sporadic HLH. This has

implications not only with respect to indication for HSCT but also with respect to the selection of a family donor, genetic counseling, and prenatal diagnosis. Health services have invested significant resources for the definitive diagnosis of these patients and provided information that will be most helpful to future patients in return. In addition, they have contributed in a unique way to our understanding of the killing mechanism through granule exocytosis and perforin, as well as shedding light on unexpected interactions among the various proteins involved.[35-37]

The breakdown of gene-specific subtypes of FHL confirms that in southern Europe FHL2 and FHL3 together account for 70% of the cases. In patients with FHL2, the W374X mutation has been previously reported as being associated with Turkish origin.[38] This is not surprising given the migratory exchanges that have occurred over 2 millennia between the Italian and Turkish populations, and it suggests that this mutation has an ancestral origin. Three patients were homozygous for A91V, a mutation present in about 5% of the European population[39-41]; for this reason, its pathogenic role has been questioned. On the other hand, it has been documented that this amino acid replacement is detrimental to the perforin protein.[30,39,42] HLH developed in 2 of our 2 homozygous patients before the child was 1 year old. The condition presented with severe liver dysfunction, perforin expression was either absent or markedly reduced (data not shown), and no mutations were found in any of the other FHL-related genes. The third patient had a full-blown HLH picture at the age of 49 years after having been given a previous diagnosis of rheumatoid arthritis. Thus we consider A91V to be pathogenic. Its high prevalence might suggest an as yet unidentified selective advantage, which deserves investigation. We observed one cluster of FHL2 in Campania and another in Sicily, but the mutations found were different.

In 50% of patients with FHL3, we found at least 1 splice defect of *UNC13D*, thus confirming the initial report by Santoro et al.[43-46] Only 1 patient (not autochthonous Italian) had the c.118-308C>T intronic mutation.[47] None of the patients carried the 253-kb inversion, which is observed in North America[48]; the c.1596+1G>C mutation, which is common in Japan[49]; or the c.754-1G>C mutation, which is present in the majority of patients with FHL3 in Korea.[50]

All the remaining genetic subtypes are represented in our geographic area, with 10% for XLP, 5% for FHL5, and a single autochthonous family with FHL4.

Sporadic cases

We have adopted the term sporadic in analogy to current use with respect to other diseases for those 56% of patients who have no family history and no evidence of a severe immunologic defect (based on a panel of tests) and in whom we did not find biallelic mutations on mutation analysis. As for clinical presentation, sporadic HLH is a phenocopy of FHL.[3,4,19] Although in sporadic cases the overall prognosis is much better than in patients with FHL, in our series 31 (11%) of them died (including 2 patients who had received HSCT) from either the disease or from treatment-related complications at a median of 29 days from diagnosis. This finding is in keeping with the report from the HLH-94 trial, in which the lack of familial disease (defined only on the basis of familial recurrence) was not associated with a significantly better outcome.[19] This implies also that sporadic HLH treatment must be prompt and must be continued until disease control has been achieved, with the reasonable expectation that reactivation is unlikely.[3,4,18,19]

Interestingly, 43 (18%) of the 240 patients with sporadic HLH had monoallelic mutations in one FHL-related gene, thus providing a tangible link between FHL and sporadic HLH. Of course, we must be cautious in interpreting this finding, which could have several explanations. First, in some patients we might have simply missed a second mutation (the finding of mutations deep in the introns of *UNC13D* has taught us a lesson[45]), in which case they would have true FHL. Second, some of these monoallelic mutations are polymorphic in the general population (eg, *PRF1*-A91V, which has been already mentioned, as well as some others). Therefore they might or might not be relevant to pathogenesis. Even if we take these 2 possibilities into account, we believe that the association with HLH of monoallelic mutations at FHL-related loci cannot be just coincidental because their frequency is much higher than could ever be observed in healthy control subjects. In addition, it stands to reason that a monoallelic loss-of-function mutation could cause an impairment in the granule exocytosis pathway or perforin expression. Indeed, on testing 28 of the 43 patients with a monoallelic mutation, we observed that none of them had a complete defect but 11 had a partial degranulation defect (17 had normal results).

Thus, as a group, patients with HLH are highly enriched for monoallelic mutations in those same genes that produce FHL when both alleles are mutated. In other words there is a gene dosage effect whereby FHL can no longer be regarded as a straightforward recessive disease. This can be explained in mechanistic terms by considering that patients with monoallelic mutations are haploinsufficient for genes involved in their perforin-dependent killing function. As yet, we are unable to provide a quantitative estimate for the risk of HLH in any person with a monoallelic mutation in one of the FHL-related genes: it is certainly less than in persons who have biallelic mutations but probably much higher than in the general population. From the practical point of view, sporadic HLH flips to FHL when another child in the same family had the disease and a member of the medical team recognizes this, which is exactly what happened in the families listed in Fig 4.

If patients with monoallelic mutations are, in a way, the missing link between FHL and sporadic HLH, perhaps we can visualize all patients with HLH as being part of a spectrum (Fig 5). We surmise that, depending on the genotype of the patient, more or less powerful triggers are required before the clinical picture of HLH explodes. In addition to the relatively frequent finding of monoallelic mutations in patients with sporadic HLH, several lines of evidence support this notion.

First, it is a given that there is always a trigger for the disease, even in those with FHL, in whom the trigger is thought to be mostly a viral infection that genetically defective cytotoxic T lymphocytes are unable to clear.

Second, although patients with FHL are prone to disease recurrence, a trigger is again required.

Third, without a trigger, the disease can be offset for years (Fig 4). Of course, because we are comparing sibs (not identical twins), modifier genes might also play a role here.

Fourth, in the HLH-94 trial patients who did not have familial disease more frequently had a recent infection.[19]

Finally, at the other end of the spectrum, hemophagocytic syndrome is a well-known if rare complication of severe bacterial, rickettsial, or protozoal infections.[17] That in such cases the

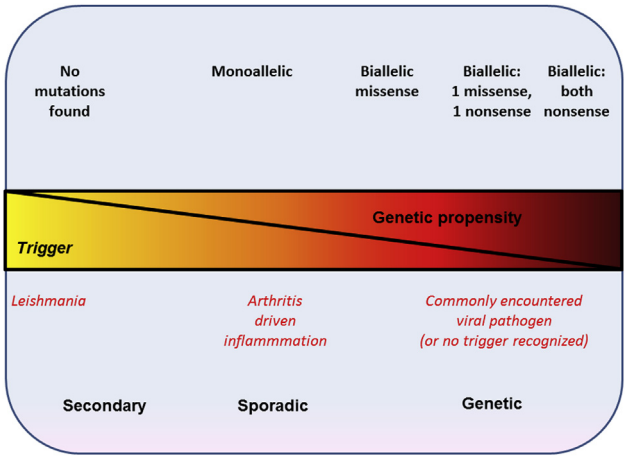

FIG 5. The risk of HLH results from the interaction of a predisposed genotype and environmental triggering factor or factors. Genotype is intended as in the FHL-related genes (*PRF1, UNC13D, STX11, STXBP2, RAB27A, LYST, SH2D1A, XIAP,* and *AP3B1*). Patients with sporadic HLH and no mutation found might harbor mutation(s) in other gene(s) currently not identified as FHL related. Selected patients might have HLH, classified as sporadic, as the presenting clinical manifestation of juvenile idiopathic systemic arthritis (MAS). Common viral pathogens can behave as a trigger for both HLH or FHL; among them, EBV or other herpesviruses are the most frequently reported.

clinical picture might resolve with appropriate specific antimicrobial therapy (and no other therapy) is good evidence that infection is indeed the trigger. Interestingly, we had 11 patients with HLH secondary to visceral leishmaniasis, and none of them had a monoallelic mutation (data not shown).

A related finding was reported recently by the Cincinnati group. Through whole-exome sequencing of DNA from patients with rheumatoid disorders who had MAS, they found variants in FHL-related genes, as well as new candidate genes,[51] and we have found that a proportion of patients with MAS harbor monoallelic mutations in FHL-related genes (manuscript in preparation). We can expect that the set of genes that predispose to HLH might expand further, particularly genes involved in inflammation.[52,53] In the meantime, it appears that patients with MAS also fit well in the spectrum illustrated in Fig 5.

Role of immunologic assays

A complete defect of perforin expression or degranulation capacity was always associated with biallelic mutations in the respective genes,[24-26] and these 2 defects were mutually exclusive (Table I). In approximately one quarter of cases of sporadic HLH, partial reduction of either perforin expression or degranulation heralded monoallelic mutation in one corresponding gene, confirming a dosage effect.[24] Another interesting question that deserves investigation is which nongenetic mechanisms might cause (down)regulation of these properties in the remaining cases presenting with a partial functional defect. Serial monitoring of sporadic cases found at diagnosis to have partial expression of perforin or impairment of degranulation is warranted and is now ongoing. At any rate, although in our hands these functional studies have proved useful to triage samples on which mutation analysis is imperative (Fig 2), we recognize that with the increase in massive sequencing, very soon mutation analysis might become the primary diagnostic approach for discriminating FHL from sporadic HLH. As a result, sophisticated assays, such as cytotoxicity and degranulation, informative on function but laborious and requiring specific expertise, might be progressively abandoned.

Diagnostic criteria might need to be updated

In our series no more than 40% of patients (data not shown) had hemophagocytosis at diagnosis. Therefore even though hemophagocytosis is a time-honored pathologic feature of HLH and is diagnostically useful when present, we must recommend that it be dropped as a diagnostic criterion. The cytotoxicity assay was used in this series in 44% of patients but in only 18% during the last 5 years; in practice it can be replaced by protein expression/degranulation assays.[24-29] It would be desirable to compare a group of patients such as this one with matched non-HLH group of patients who have septicemia or inflammatory diseases to achieve an unbiased assessment of old and new diagnostic tests. The time might have come for a consensus conference that will update recommendations regarding the most appropriate diagnostic tests.

In conclusion, while giving credit to Bodley-Scott and Robb-Smith for their seminal work on HLH,[1] we must state today that the ultimate diagnosis of this disease is not through microscopy. These authors could not have surmised that there is a strong genetic component in at least a substantial proportion of cases, and our data show clearly that the diagnosis must be based on a combination of targeted molecular analysis and specific functional studies of lymphocytes. We have shown that by detecting biallelic mutations, we can promptly identify patients with FHL and that children with HLH during the first year of life have a strikingly high probability to have FHL, with FHL2 and FHL3 being the most frequent genetic subtypes in southern Europe. It is gratifying that with earlier accurate diagnosis, the prognosis has tangibly improved. In our systematic analysis of 500 cases of HLH, we have found monoallelic mutations in FHL-related genes in 15% of cases of sporadic HLH: this must mean that these mutations can make the function of the other allele inadequate in the face of certain challenges (haploinsufficiency). Whether we classify these cases as familial or sporadic becomes a matter of semantics, but clearly, we can no longer simply say that FHL is recessive. At the same time, one wonders whether patients with sporadic HLH in whom we find no mutation in FHL-related genes might have mutations in other genes, such as those that control the production or secretion of IFN-γ. This question should be soon settled by using full-exome sequencing.

In the meantime, it appears reasonable to surmise that the explosion of HLH is like being pushed over the edge into a potentially catastrophic drop. If the push (eg, an infectious trigger) is powerful enough, anybody can be made to fall, but if the edge is lowered (certain genes are mutated), a much weaker push might be sufficient to produce the same result. This will be true of persons with a monoallelic mutation and even more of those with biallelic mutations.

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Pediatrica Ospedale dei Bambini G. Salesi (P. Pierani); Bari, Dipartimento Biomedicina Età Evolutiva U.O. Pediatrica I Policlinico (N. Santoro); Bergamo, Ospedale Papa Giovanni XXIII U.O. Pediatria, Sezione Oncoematologia Pediatrica (M. Provenzi); Bologna, Oncologia ed Ematologia Pediatrica "Lalla Seragnoli" Clinica Pediatrica Policlinico Sant'Orsola Malpighi (A. Pession); Brescia, Clinica Pediatrica oncoematologia pediatrica e TMO Ospedale dei Bambini (F. Porta); Firenze, Dipartimento A.I. Oncoematologia, SODC Tumori Pediatrici e Trapianto di cellule staminali, A.O.U Meyer (C. Favre); Genova, Dipartimento di Ematologia e Oncologia Pediatrica, Istituto G.Gaslini (F. Frassoni); Monza, Clinica Pediatrica Università Milano–Bicocca, A.O. San Gerardo–Fondazione MBBM (A. Biondi); Napoli, Dipartimento di Oncologia, A.O. Santobono-Pausilipon (V. Poggi); Palermo, U.O Oncoematologia Pediatrica, ARNAS Civico Di Cristina e Benfratelli (P. D'Angelo); Padova, Dipartimento di Pediatria, Università di Padova, Cattedra di Oncoematologia Pediatrica (G. Basso); Pavia, Oncoematologia Pediatrica, Fondazione Irccs, Policlinico San Matteo (M. Zecca); Roma, Oncoematologia Pediatrica, Ospedale Bambino Gesù (F. Locatelli); and Torino, S.C. Oncoematologia Pediatria e Centro Trapianti, Ospedale Infantile Regina Margherita (F. Fagioli).

Clinical implications: The analysis of 500 patients with HLH enrolled in the last 25 years could help redefine the clinical picture of this condition and revise the diagnostic approach.

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METHODS

Diagnostic strategy in patients with HLH

Immunologic tools have been developed to pinpoint rapidly those patients who have the most frequent defects underlying FHL to speed up the procedure of discrimination between sporadic cases and cases with biallelic mutations. Flow cytometric analysis of peripheral blood lymphocytes allows detection of defective intracytoplasmic perforin expression and SAP and XIAP expression and reduced capacity of the stimulated cytotoxic lymphocytes to "degranulate," as measured by induced surface expression of the CD107a molecule.[E1-E4] Additional tools have been developed to investigate SAP and XIAP expression in male patients with suspected HLH-related conditions.[E5,E6] Detection of defective cellular cytotoxicity, although included in the diagnostic criteria, requires the use of radionuclides, which makes it impractical; thus its use has been progressively restricted to investigation of highly selected patients, mostly for research purposes. Because the above methods require specific skills, regional reference laboratories have been developed.[E7]

Immunologic analyses

Protein expression, degranulation, cytotoxicity assays, and mutation analysis were performed, as previously described.[E1,E2,E4-E7] PBMCs from patients and healthy donors were isolated by means of Ficoll gradient centrifugation. NK cells were also purified by 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 μg/mL PHA (Sigma-Aldrich, Irvine, United Kingdom) and 600 IU/mL rIL-2 (Proleukin; Chiron, Emeryville, Calif) to obtain high numbers of polyclonal activated NK cell populations.

Perforin expression on NK cells ($CD3^-CD56^+$ cells of peripheral blood lymphocyte or purified activated NK populations) was detected by means of intracellular staining (after fixation and permeabilization) with δG9 mAb (IgG_{2b}) and cytofluorimetric analysis, as previously reported.[E2] Intracellular SAP expression with the 1C9 mAb (IgG_{2a}; Abnova, Taipei, TW) and staining with anti-XIAP (IgG_1; BD Bioscience, San Jose, Calif) were evaluated, followed by phycoerythrin-conjugated isotype specific second reagents (SouthernBiotech, Birmingham, Ala). Mouse isotype-matched control mAbs (BD Bioscience) were used.[E5,E6]

To analyze the cytolytic activity in 4-hour ^{51}Cr-release assays, PBMCs were tested against K562 cells and activated NK cells were tested against the HLA class I^- B-EBV cell line 721.221, which were demonstrated to be suitable effector/target combinations to reveal cytolytic defect of patients with FHL. Effector cell/target cell ratios ranging from 100:1 to 1:1 were used for PBMCs as effector cells, whereas ratios ranging from 8:1 to 0.5:1 were used for activated NK cells. Lytic units at 30% lysis were calculated. Resting and activated NK cells were also tested in degranulation assays quantifying cell-surface CD107a expression on coculture with K562 cells, as previously described.[E3] Briefly, anti-CD107a–phycoerythrin mAb was added during the coculture for 3 hours at 37°C in a 5% CO_2 atmosphere. Thereafter, the cells were stained with anti-CD56–allophycocyanin and anti-CD3–peridinin-chlorophyll-protein complex mAbs and analyzed by using flow cytometry (FACSCalibur, Becton Dickinson). All reagents were from BD Biosciences. Surface expression of CD107a was assessed in the $CD3^-CD56^+$ cells. Results were evaluated as ΔCD107a (ie, Percentage $CD107a^+$ cells of stimulated samples − Percentage of $CD107a^+$ cells of unstimulated samples) and defined as defective when lower than the tenth percentile of healthy control subjects. 2B4 function was evaluated in degranulation or ^{51}Cr release assays by using either PBMCs short-term cultured with rIL-2 or activated NK cells as effectors in reverse antibody-dependent cellular cytotoxicity against the P815 ($Fc\gamma Rc^+$) target cell line in the presence of functional grade purified anti-2B4 mAb (PP35; eBioscience, San Diego, Calif) alone or in combination with the anti-NKp46 mAb (9E2; Miltenyi Biotec, Bergisch Gladbach, Germany; or BAB281 cells).[E5,E6]

Mutation analysis

Genomic DNA was isolated from peripheral blood samples by using the BioRobot EZ1 Workstation (Qiagen, Jesi, Italy) or QIAamp DNA Mini Kit (Qiagen). *PRF1* (OMIM *170280), *UNC13D* (*608897), *STXBP2* (*601717), and *STX11* (*605014) have been analyzed in all patients. Male patients were also tested for *SH2D1A* (*300490) and *XIAP* (*300079). Where pigment deficiency was reported, samples underwent *RAB27A* (*603868) or *LYST* (*606897) gene analysis. Briefly, coding exons and exon-intron boundaries of the FHL-related genes were amplified and directly sequenced in both directions with the BigDye Terminator Cycle SequencingReady Reaction Kit (Applied Biosystems, Foster City, Calif). Amplification reactions were performed with 60 ng of DNA, 10 ng of each primer, 200 μmol/L dNTPs, 1× PCR reaction buffer, and 2.5 U of Taq polymerase in a final volume of 25 μL. Sequences obtained with an ABI Prism 3130XL Sequence Detection System (Applied Biosystems) were analyzed and compared with the reported gene structure by using the dedicated software SeqScape (Applied Biosystems). All mutations were confirmed in the parents and tested in all available family members on request.

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TABLE E1. Revised diagnostic guidelines for HLH[E7]

The diagnosis of HLH can be established if either 1 or 2 of the following are fulfilled:
1. A molecular diagnosis consistent with HLH (ie, biallelic mutations in 1 FHL-related gene)
2. Clinical and laboratory criteria for HLH fulfilled (≥5 of the following 8 criteria fulfilled):
Fever
Splenomegaly
Cytopenia (affecting ≥2 of 3 lineages in peripheral blood):
Hemoglobin <9 g/dl (in infants <4 wk: Hb <10 g/dL)
Platelets $<100 \times 10^9$/L
Neutrophils $<1.0 \times 10^9$/L
Hypertriglyceridemia and/or hypofibrinogenemia
Fasting triglycerides ≥3.0 mmol/L
Fibrinogen ≤1.5 g/L
Hemophagocytosis in bone marrow or spleen or lymph nodes
Low or absent NK cell activity
Ferritin ≥500 μg/L
Soluble CD25 (ie, soluble IL-2 receptor) ≥2400 U/mL
Additional features, such as:
• Cerebral symptoms with moderate pleocytosis and/or increased protein levels in cerebrospinal fluid
• Increased transaminase levels
• Increased bilirubin levels
• Increased lactate dehydrogenase levels
Might be present in a minority of cases and thus their presence might contribute to suspicion of HLH.

TABLE E2. Number of cases of HLH reported to the Italian National registry by time interval

Time interval	Total no. of patients (mean no. per year)	Patients with biallelic mutation (mean no. per year)	Sporadic patients (mean no. per year)	Biallelic/sporadic ratio
1989-1994*	53 (8.8)	28 (4.6)	25 (4.1)	1.12
1995-1999	46 (9.2)	28 (5.6)	18 (3.6)	1.55
2004-2004	67 (13.4)	32 (6.4)	35 (7.0)	0.91
2005-2009	127 (25.4)	37 (7.5)	90 (18.0)	0.41
2010-2014†	207 (48.7)	48 (11.2)	159 (37.4)	0.30
Total	500	173	327	0.52

Note: The frequency is corrected according to the observation times as follows: *6 years for the initial cohort; †4.3 years for the most recent cohort ending March 31, 2014. The current total number of inhabitants in the country is 59,685,227, including 521,855 newborns in 2013 (http://demo.istat.it/pop2013/index.html).

TABLE E3. Main features of 15 patients given a diagnosis of FHL based on clinical or immunologic findings in which mutations were not identified in any of the FHL-related genes

Unique patient no.	Origin	Consanguinity	Familial disease	Sex/age	Clinical course	Functional studies
86*	Italy	Yes	Yes	M/1.4	Dead of progressive disease	Not performed
176	Italy	No	Yes	M/0.4	Dead of progressive disease	Absent NK activity
186*	Italy	Yes	Yes	M/0.8	Dead of surgical complication, before transplantation	Normal NK activity
331	Italy	No	No	F/1.1	Recurrent disease, severe encephalopathy	Normal perforin expression, GRA, and NK activity
347	Italy	No	No	F/2.1	Cured after transplantation	Normal perforin expression, reduced GRA, defective NK activity
456†	Italy	No	Yes	M/3.1	Dead of progressive disease	Not performed
524†	Italy	No	Yes	F/3.4	Cured after transplantation	Normal perforin expression, reduced GRA, absent NK activity
540	Italy	No	No	F/11.7	Dead of progressive disease	Normal perforin expression, reduced GRA, normal NK activity
587	Pakistan	Yes	Yes	M/0.7	Dead of progressive disease	Normal perforin expression, normal GRA
608	Italy	No	No	F/3.8	Dead of progressive disease	Normal perforin expression, GRA, and NK activity
690	Italy	No	No	M/0.2	Recurrent disease, severe encephalopathy	Normal perforin expression and GRA
696	Italy	No	No	M/0.7	Recurrent disease; dead of complication after transplantation	Normal perforin expression and GRA; NK activity mildly reduced
728	Asia	No	No	F/2.4	Dead of progressive disease	Normal perforin expression and GRA; NK activity reduced
756	Italy	No	No	M/2.4	Dead of progressive disease	Not Performed
797	Italy	No	No	M/1.7	Dead of progressive disease	Normal perforin expression and GRA

F, Female; *GRA*, granule release assay; *M*, male.
*Unique patient numbers 86 and 186 are siblings.
†Unique patient numbers 456 and 524 are siblings.