Severe adult hemophagocytic lymphohistiocytosis (HLHa) correlates with HLH-related gene variants

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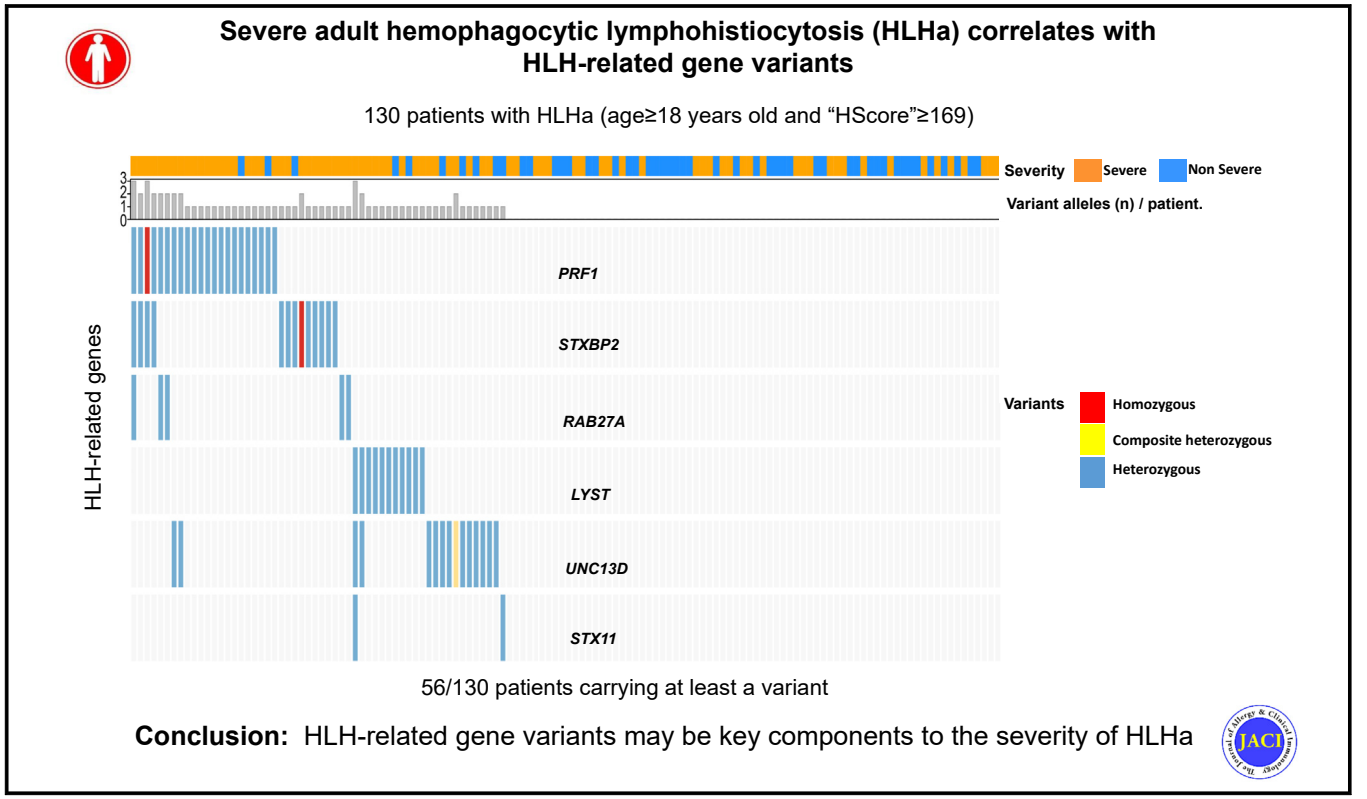
GRAPHICAL ABSTRACT



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Background: The contribution of genetic factors to the severity of adult hemophagocytic lymphohistiocytosis (HLHa) remains unclear.

Objective: We sought to assess a potential link between HLHa outcomes and HLH-related gene variants.

Methods: Clinical characteristics of 130 HLHa patients (age ≥ 18 years and HScore ≥ 169) and genotype of 8 HLH-related genes (*LYST*, *PRF1*, *UNC13-D*, *STX11*, *STXBP2*, *RAB27A*, *XIAP*, and *SAP*) were collected. A total of 34 variants found in only 6 genes were selected on the basis of their frequency and criteria predicted to impair protein function. Severity was defined by refractory disease to HLH treatment, death, or transfer to an intensive care unit.

Results: HLHa-associated diseases (ADs) were neoplasia (n = 49 [37.7%]), autoimmune/inflammatory disease (n = 33 [25.4%]), or idiopathic when no AD was identified (n = 48 [36.9%]). Infectious events occurred in 76 (58.5%) patients and were equally distributed in all ADs. Severe and refractory HLHa were observed in 80 (61.5%) and 64 (49.2%) patients, respectively. HScore, age, sex ratio, AD, and infectious events showed no significant association with HLHa severity. Variants were identified in 71 alleles and were present in 56 (43.1%) patients. They were distributed as follows: 44 (34.4%), 9 (6.9%), and 3 (2.3%) patients carrying 1, 2, and 3 variant alleles, respectively. In a logistic regression model, only the number of variants was significantly associated with HLHa severity (1 vs 0: 3.86 [1.73-9.14], $P = .0008$; 2-3 vs 0: 29.4 [3.62-3810], $P = .0002$) and refractoriness (1 vs 0: 2.47 [1.17-5.34], $P = .018$; 2-3 vs 0: 13.2 [2.91-126.8], $P = .0003$).

Conclusions: HLH-related gene variants may be key components to the severity and refractoriness of HLHa. (J Allergy Clin Immunol 2024;153:256-64.)

Key words: *Hemophagocytic lymphohistiocytosis (HLH), HLH-related gene variants, adult secondary HLH, severe HLH in adults*

Hemophagocytic lymphohistiocytosis (HLH) syndrome is caused by abnormal and sustained activation of T lymphocytes and macrophages, releasing proinflammatory cytokines leading to cytokine storm syndrome.[1,2] HLH is characterized by multiple clinical and biological features, including fever, splenomegaly, high ferritin level, pancytopenia, and liver biology abnormalities. HLH is commonly divided into primary/familial (F-HLH) cases and secondary/acquired cases.[2,3] F-HLH is a severe disease occurring mainly in children at an early age (usually <3 years).[4] F-HLH occurrence is linked to recessive-autosomal or X-linked mutation in any of the following genes: *PRF1*,[5] *UNC13-D*,[6] *STX11*,[7] *STXBP2*,[8] *RAB27A*,[9] *LYST*,[10] *SH2D1A*,[11] and *XIAP*.[12] Except for *XIAP* mutations, HLH-related gene mutations cause a functional deficiency of the perforin-dependent cytotoxic pathway of CD8 T lymphocytes and natural killer cells.[13,14]

Acquired/secondary HLH is generally considered not genetically linked, being sporadic with no hints of familial history and occurring mostly in adults but also in adolescents and children, usually at an older age than in F-HLH.[15,16] It usually appears in the context of an underlying disease, which most frequently is neoplasia[17,18] or autoimmune/inflammatory disease (AID). In this context, HLH is called macrophage activation syndrome. In some cases, no associated disease (AD) is found, and HLH is then classified as idiopathic. However, in most cases, regardless of the underlying disease, an infection triggers HLH onset.[19,20]

HLH outcome is highly polymorphic, ranging from benign manifestations to severe forms requiring intensive care and leading to death in 60% of cases.[21,22] Malignancy ADs are commonly associated with a poor prognosis,[23-25] but other factors contributing to the clinical heterogeneity have not been well deciphered.

Although there is a fair amount of evidence linking heterozygous missense mutations in F-HLH genes with adult HLH (HLHa),[26-28] it is still considered to be mainly determined by external stressors,[26,29-31] and the potential value of variants in HLH-related genes in the severity of HLH has never been addressed.

Here, we investigate a cohort of HLHa patients genotyped for 8 HLH-related genes to assess possible links with clinical outcomes.

Abbreviations used
AD: Associated disease
AID: Autoimmune/inflammatory disease
F-HLH: Familial HLH
HLH: Hemophagocytic lymphohistiocytosis
HLHa: HLH in adults
ICU: Intensive care unit

METHODS

Selection of patients

Between 2012 and 2019, 195 patients (178 adults, 6 adolescents, and 11 children 3-11 years old) with secondary HLH were prospectively recruited from 62 medical centers in a national study (funded by the French National Program of Clinical Research, 2010). The inclusion criteria were adapted from the HLH-2004 diagnostic criteria[32] and with the expertise of the French HLH Study Group, as recommended[33] (see Table E1 in this article's Online Repository at www.jacionline.org). Informed consent was obtained from all study participants following the Declaration of Helsinki. The regional Ethics Committee of Paris (ID-RCB: 2009-AO00301-56) approved the study. The data collected included clinical and biological features of HLH, ADs, infectious trigger events, treatment, and outcomes (refractoriness to treatment [corticosteroids and etoposide] or relapse, intensive care unit [ICU] transfer, and death) at HLH onset and during a 1-year follow-up. Serum and DNA samples were

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obtained for each patient. Selected ADs included neoplastic diseases (hematologic malignancies and solid tumors) or AID. The patients were classified as idiopathic if they did not have a history or the occurrence of neoplasia and/or AID during the 1-year follow-up of the study. The diagnosis of AID was based on the American College of Rheumatology criteria for systemic lupus erythematosus[34] or on the Yamaguchi criteria for Still disease.[35] Bacterial, viral, parasitic, or miscellaneous infectious events were considered potential triggers of HLH onset and were collected. Treatment of HLH was noted, particularly the use of corticosteroids and etoposide, which are recommended in severe forms of HLH.[36] Independently and before genetic studies, patients were classified as "severe HLH" when he or she experienced at least 1 of the following events: transfer to the ICU, refractory disease to treatment (refractory to the first line of treatment leading to a second line of treatment or relapse), or death.

Between 2014 and 2016, 172 patients were sequenced for 8 selected genes (Fig 1) and referred to as the "HLH-related genes cohort." The following genes involved in F-HLH and belonging to the same cytotoxic physiological pathway were considered: *PRF1*, *UNC13-D*, *STX11*, *STXBP2*, *RAB27A* (Griscelli syndrome [*GS2* gene]), and *LYST* (Chediak-Higashi syndrome). The genes involved in X-linked lymphoproliferative diseases 1 and 2, *SH2D1A/SAP* and *BIRC4/XIAP*, were also retained for sequencing.

On the day of enrollment, HScore and the diagnostic values of the HLH-2004 criteria were calculated for the 172 patients in the HLH-related genes cohort. Because HScore[37] is reported to have a better prediction accuracy than the HLH-2004 criteria for HLHa,[38-40] patients of the HLH-related genes cohort (1) older than 18 years and (2) with an HScore greater than or equal to 169[37] (Fig 1) were classified as HLHa. Overall, 130 patients fulfilled these 2 criteria (median HScore, 218 [169-320]) and composed the HLHa-related genes cohort. Forty-two patients were excluded from this study: 12 because of being younger than 18 years and 30 because of an HScore of less than 169 (Fig 1). Of note, 113 (87%) of the 130 HLHa patients had fulfilled at least 4 HLH-2004 criteria.[32,40]

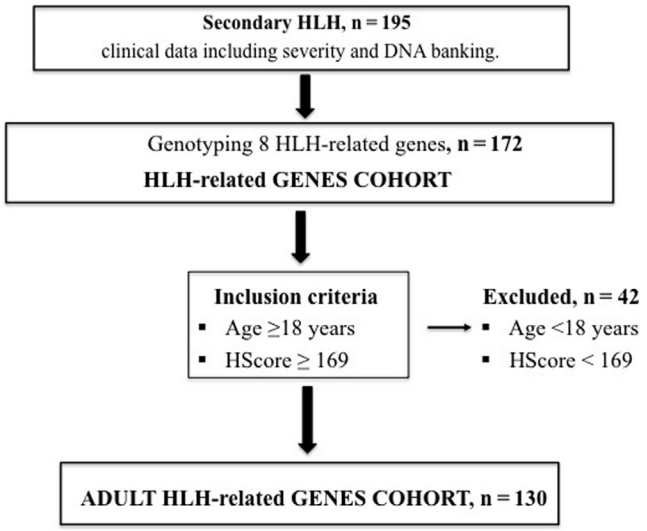

FIG 1. Flow chart of the HLHa genes cohort.

Selection of HLH-related gene variants

Targeted gene sequencing methods are detailed in this article's Online Repository at www.jacionline.org.

We identified 271 variants localized in the capture regions of the 8 targeted genes (HLH-related genes). We excluded intronic variants because of incomplete coverage of the intronic regions by the capture panel as well as splicing and other noncoding variants because they are not scored by PolyPhen and incompletely by CADD. After the exclusion of intronic (n = 138), UTR3 (n = 5), UTR5 (n = 2), and splicing (n = 14) variants, as well as synonymous variants (n = 53), a total of 59 nonsynonymous variants were retained for further analyses, all of them being missense and exonic.

Finally, variants were subsequently selected if they met at least 2 of the following criteria: (1) the maximum minor allelic frequency in reference subpopulations is less than 0.05 (ANNOVAR PopFreqMax annotation), (2) CADD phred score is greater than or equal to 14.399, and (3) PolyPhen-2 HVAR score is greater than or equal to 0.429 according to the Mutational Significance Cutoff database thresholds[41] (see Fig E1 in this article's Online Repository at www.jacionline.org). Thus, 33 variants that fulfilled these criteria were selected (Fig E1). Among them, the *PRF1* A91V variant, previously related to F-HLH,[42,43] met the frequency and the 2 pathogenicity criteria. One more variant, *PRF1* N252S, despite meeting only the frequency criterion, was also selected because of being of potential clinical significance in ClinVar annotation and was related to F-HLH.[43,44] Finally, 34 variants were selected for analysis, including 14 variants fulfilling all the 3 criteria, 18 with only 2 criteria, and 2 with ClinVar annotation (Fig E1; see Table E2 in this article's Online Repository at www.jacionline.org). Notably, no variants were identified in *SH2D1A/SAP* and *BIRC4/XIAP* genes.

Statistical methods

Characteristics were summarized for quantitative data by the median (minimum-maximum values) and group comparisons performed using the Wilcoxon rank-sum test. Qualitative data were summarized by frequencies (percentages) and compared using the Fisher exact test. Exact Cochran-Armitage trend tests were used to test the dependence between ordered and binary variables.

To compare collapsed variant allelic frequencies between HLHa-related genes and in-house control cohorts (see the Online Repository), Cohort Allelic Sums Test (CAST) and SUM burden tests adjusted for population stratification by Principal Component Analysis (PCA) were used (see the Online Repository). PCA-adjusted burden tests are a common and efficient strategy for accounting for different ancestries in patients and controls in the study of rare variants.[45,46]

Stepwise multivariate Firth bias-reduced logistic regression models[47] were fitted to select clinical and genetic characteristics associated with HLH severity. Adjusted odds ratios were estimated from these models and were reported with their 95% CI computed by the profile likelihood method. Statistical tests were defined as 2-tailed, and P values less than .05 were

TABLE I. Clinical characteristics of HLHa patients according to AD

Characteristics	Overall (n = 130)	Neoplasia (n = 49 [37.7%])	AID (n = 33 [25.4%])	Idiopathic (n = 48 [36.9%])
HScore at diagnosis, median (range)	218 (169-320)	226 (174-320)	203 (172-274)	225 (169-310)
Sex ratio (male/female)	1.3 (74/56)	1.7 (31/18)	0.6 (12/21)	1.8 (31/17)
Age at diagnosis (y), median (range)	50 (18-83)	56.0 (31-83)	41.0 (21-81)	44.5 (18-81)
Infectious triggers, n (%)				
Yes	76 (58.5)	23 (46.9)	21 (63.6)	32 (66.7)
EBV*	38 (29.0)	19 (38.8)	15 (44.1)	8 (16.7)
Herpes virus no EBV	11 (8.4)	1 (2.0)	1 (3.0)	5 (10.4)
Intracellular bacteria	15 (11.4)	0 (0.0)	2 (5.9)	13 (27.1)
Other pathogens (no Herpes virus, bacteria, or parasites)	12 (9.2)	3 (6.1)	3 (8.8)	6 (12.5)
No	54 (41.5)	26 (53.1)	12 (36.4)	16 (33.3)
Outcomes, n (%)				
Transfer to ICU	42 (32.3)	14 (28.6)	12 (36.4)	16 (33.3)
Refractory to treatment†	64 (49.2)	29 (59.2)	15 (45.5)	20 (41.7)
Death	31 (23.8)	20 (40.8)	6 (18.2)	5 (10.4)

*PCR EBV virus load detected by PCR.
†Treatment: corticosteroid, etoposide.

considered statistically significant. All computations were performed with the R-Bioconductor statistical environment V3.6 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Clinical features of the HLHa-related genes cohort

The HLHa-related genes cohort gathered 130 patients. None had a familial history of HLH. The sex ratio (male/female) was 1.3 (74/56), and the median age at diagnosis was 50 years (range, 18-83 years). Infectious triggers were reported in 58.5% (n = 76) of patients and were classified into 4 main categories: (1) EBV, (2) non-EBV herpes virus group, (3) intracellular bacteria, and (4) others (Table I). HLHa-ADs were diagnosed as neoplastic diseases (n = 49 [37.7%]), AIDs (n = 33 [25.4%]), or idiopathic when no AD was diagnosed (n = 48 [36.9%]). Transfer to ICU, refractory disease, or death occurred in 42 (32.3%), 64 (49.2%), and 31 (23.8%) patients, respectively, within 1 year following diagnosis (Table I). Among the 130 HLHa cases, 80 (61.8%) patients presented at least 1 of the severity criteria and were classified as severe (Table II). Severe and refractory patients were more frequently treated with etoposide compared with nonsevere patients (40 of 80 [50%] vs 5 of 50 [10%], $P < 1.5 \times 10^{-6}$; 35 of 64 [54%] vs 10 of 64 [15.6%], $P < 2.68 \times 10^{-6}$) (Table II; see also Table E3 in this article's Online Repository at www.jacionline.org).

When comparing the distribution of the clinical parameters between patients exhibiting severe and nonsevere features, no statistical differences were found in the distribution of HScore, age at diagnosis, sex ratio, infectious triggers, and ADs (Table II). In particular, the frequency of EBV replication was comparable in both groups (29.6% vs 28%). Although not reaching statistical significance ($P = .095$), neoplasia was more frequently observed in patients with severe disease (43.8% vs 28%) (Table II).

Distribution of HLH-related gene variants in HLHa-related genes cohort and correlation with clinical features

As shown in Fig 2, 34 selected variants were distributed in 71 alleles, which were present in 56 (43.1%) patients of the cohort. All the variants were heterozygous except for 1 homozygous *PRF1* variant and 1 *STXBP2* variant. Heterozygous composite variants were found in *UNC13D*. The 2 *PRF1* variants, A91V and N252S, were the most frequently observed (13 alleles in 12 patients and 8 alleles in 8 patients, respectively) (Fig 2). A monoallelic variant was detected in 44 (34.4%) patients (see Table E4 in this article's Online Repository at www.jacionline.org). Polyallelic distribution was found in 12 patients, of whom 9 (6.9%) and 3 (2.3%) patients carried 2 and 3 variant alleles, respectively (see Table E5 in this article's Online Repository at www.jacionline.org). Of note, combinations of *PRF1/STXBP2*, *PRF1/RAB27A*, and *PRF1/STXBP2/RAB27A* gene variants were observed in 4, 3, and 1 patient, respectively (Table E5).

A PCA-adjusted burden test on the 6 HLH-related genes revealed an enrichment in variant alleles in the overall cohort (53%) relative to controls (40%) (PCA-adjusted CAST test, $P = .027$; PCA-adjusted SUM test, $P = .013$) (see Table E6 in this article's Online Repository at www.jacionline.org).

Association of HLH-related gene variants with HLHa clinical features

None of the clinical features, including sex ratio, age, ADs (neoplasia, AID, or idiopathic), or infectious events, were significantly associated with the number of variant alleles (see Tables E7 and E8 in this article's Online Repository at www.jacionline.org). Severe HLHa patients more frequently carried at least 1 variant allele (46 of 80 [57.7%]) compared with others (10 of 50 [20%]) (Fisher test, $P = 2.6 \times 10^{-5}$). Considering the number of variant alleles per patient, we detected a positive trend between the number of variant alleles found and the severity of the HLHa (exact Cochran-Armitage trend test, $P = 1.0 \times 10^{-5}$) (Table III). Of note, the 12 patients carrying 2 or 3 variant alleles were all severe. Conversely, none of the nonsevere patients carried more than 1 variant allele ($P = 6.1 \times 10^{-6}$). The same relationship with severity was found when separately considering the *PRF1* A91V variant (11 of 80 for severe patients vs 1 of 50 for nonsevere patients; Fisher test, $P = .028$; trend test, $P = .047$) from the other variants (41 of 80 for severe patients vs 9 of 50 for nonsevere patients; Fisher test, $P = 1.8 \times 10^{-4}$; trend test, $P = 1.2 \times 10^{-4}$) (Table III).

TABLE II. HLHa clinical features associated with HLH severity

Features	Severity		*P* value
	Severe (n = 80 [61.8%])	Nonsevere (n = 50 [38.2%])	
Etoposide treatment, n (%)	40 (50)	5 (10)	1.5×10^{-6}
HScore at diagnosis, median (range)	224 (174-320)	213 (169-294)	.14
Sex ratio (male/female)	1.4 (47/33)	1.2 (27/23)	.72
Age at diagnosis (y), median (range)	51 (19-83)	48 (18-73)	.13
Infectious triggers, n (%)			
EBV	24 (29.6)	14 (28.0)	.90
Herpes virus non-EBV	6 (7.4)	5 (10.0)	
Intracellular bacteria	8 (9.9)	7 (14.0)	
Other pathogens	7 (8.6)	5 (10.0)	
No	35 (44.5)	19 (38.0)	.59*
AD, n (%)			
AID	19 (23.8)	14 (28.0)	.18
Idiopathic	26 (32.4)	22 (44.0)	
Neoplasia	35 (43.8)	14 (28.0)	.09†

*Fisher exact test (infectious trigger: yes vs no).
†Fisher exact test (neoplasia vs AID + idiopathic AD).

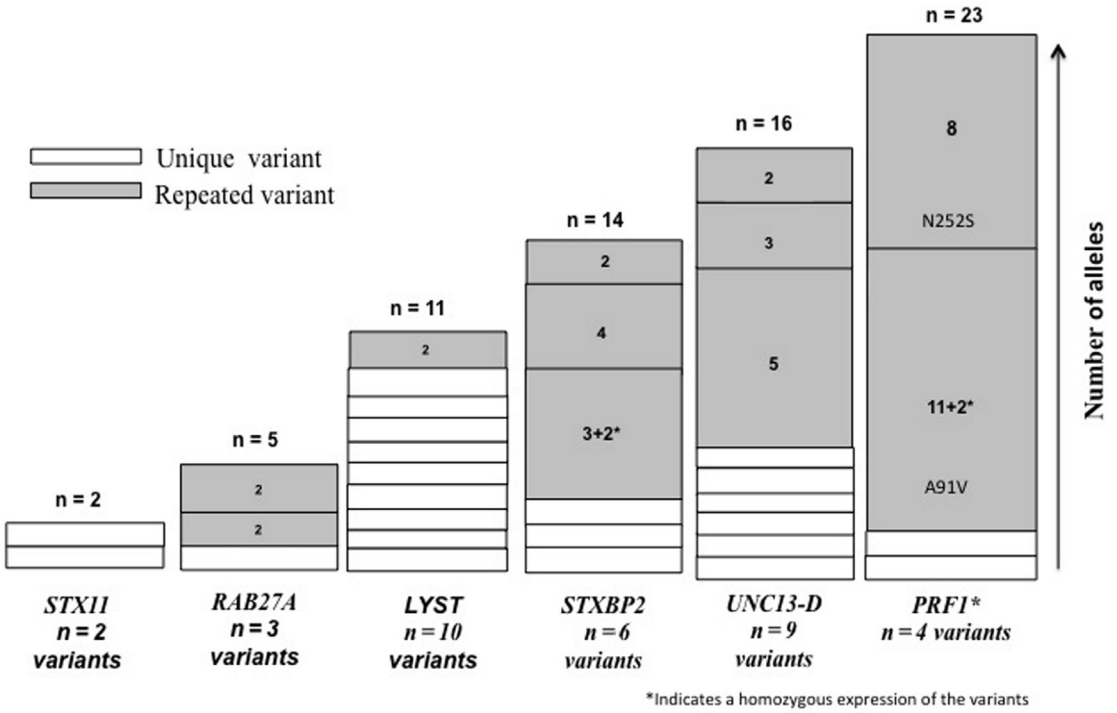

FIG 2. Allelic distribution of variants detailed for each HLH gene in HLHa. Each box is an allele carrying a unique (*white box*) or repeated (*gray box*) allele variant. For each HLH-related gene, the number of alleles carrying unique or repeated variants is provided. The number in the box indicates the number of alleles recurrency. *Indicates a homozygous expression of the variants.

In a penalized stepwise logistic regression, including the clinical characteristics and the number of variant alleles in the 6 genes, only the genetic factor was significantly associated with severity (penalized likelihood ratio test, $P = 2.0 \times 10^{-5}$). The odds ratio estimate for 2 to 3 alleles versus 0 comparisons (29.4, 95% CI [3.62; 3810.9]) was higher than the estimate for 1 variant allele versus 0 comparisons (3.86, 95% CI [1.73; 9.14]) (Table IV). When *PRF1* A91V variant alleles were excluded, results were similar (5.57, 95% CI [1.20; 53.71]) (1 allele vs 0: 3.79, 95% CI [1.66; 9.26]; 2-3 alleles vs 0: 15.4, 95% CI [1.72; 2037.4]) (Table IV). When separately analyzing the 3 components of severity (refractoriness, ICU transfer, and death), it appears that HLHa refractoriness was the only parameter associated with the presence of variant alleles. Indeed, 37 (58%) of 64 refractory patients carried at least 1 variant allele versus 19 (29%) of 66 for no refractoriness (Fisher test, $P = 1.3 \times 10^{-3}$) (see Table E9A in this article's Online Repository at www.jacionline.org). The trend test between the number of

TABLE III. Variant alleles frequencies in severe and nonsevere patients

Variant	No. of alleles	Severe (%) (n = 80)	Nonsevere (%) (n = 50)	*P* value
All variants	0	34 (42.5)	40 (80.0)	1.0×10^{-5}*
	1	34 (42.5)	10 (20.0)	6.1×10^{-6}†
	2	9 (11.2)	0	2.6×10^{-5}‡
	3	3 (3.8)	0	
PRF1 A91V	0	69 (86.2)	49 (98.0)	4.7×10^{-2}*
	1	10 (12.5)	1 (2.0)	2.8×10^{-2}‡
	2	1 (1.3)	0	
Other variants	0	39 (48.8)	41 (82.0)	1.2×10^{-4}*
	1	35 (43.7)	9 (18.0)	7.0×10^{-5}†
	2	4 (5.0)	0	1.8×10^{-4}‡
	3	2 (2.5)	0	

*Exact trend test *P* value on all alleles number classes.
†Exact trend test *P* value by collapsing the last 2 alleles number classes.
‡Fisher exact test *P* value, 0 vs >0 alleles number classes.

TABLE IV. Logistic regression, with all variant alleles and *PRF1* A91V–excluded variant alleles, association with severity of HLH

Variant	No. of alleles	Odds ratio	95% CI*	*P* value
All variants†				
	0	1.00		
	1	3.86	1.73-9.14	.0008
	2-3	29.4	3.62-3810.9	.0002
PRF1 A91V + other variants‡				
	0	1.00		
PRF1 A91V	1-2	5.57	1.20-53.71	.0265
Other variants	1	3.79	1.66-9.26	.0013
	2-3	15.4	1.72-2037.4	.0099

df, Degree of freedom.
*Determined by penalized profile likelihood method.
†Penalized likelihood ratio test, 20.93 on 2 df; $P = 2.9 \times 10^{-5}$.
‡Penalized likelihood ratio test, 20.12 on 3 df; $P = 1.6 \times 10^{-4}$.

alleles and refractoriness was also significant (exact Cochran-Armitage trend test, $P = 2.0 \times 10^{-4}$), and similar relationships with *PRF1* A91V variant (11 of 64 for severe patients vs 1 of 66 for nonsevere; Fisher test, $P = 2.0 \times 10^{-3}$; trend test, $P = 1.8 \times 10^{-3}$) and other variants (32 of 64 for severe patients vs 18 of 66 for nonsevere; Fisher test, $P = 1.0 \times 10^{-2}$; trend test, $P = 1.0 \times 10^{-2}$) were also observed (Table E9A).

Logistic models confirm that only the genetic components were associated with refractoriness, considering either the whole variant alleles (penalized likelihood ratio test, $P = 4.0 \times 10^{-4}$) or excluding A91V (penalized likelihood ratio test, $P = 5.0 \times 10^{-4}$), with an increase in odds ratio estimates between monoallelic versus multiallelic variants (Table E9B). However, no relationship was found between the detection of variant alleles and ICU or death outcome (see Table E10A and B in this article's Online Repository at www.jacionline.org). In our adult cohort, death was primarily associated with the underlying neoplasia (20 of 31 vs 29 of 99, $P = .0006$) and the age of the patients (death: 57 [23-83] vs 47 [18-82], $P = .003$).

DISCUSSION

This study aimed to test the hypothesis that heterogeneity of HLHa outcome may have a genetic basis. We took advantage of a national cohort with clinical features prospectively characterized to perform genotyping of 8 HLH genes.

We have selected 34 HLH-related gene variants in this cohort, including 2 linked to F-HLH (*PRF1* A91V and N252S). We found that 43.1% (56 of 130) of patients carried at least 1 variant allele, and 12 carried polyallelic variants. By using PCA-adjusted burden tests, we found a global enrichment of HLH gene variants compared with the control in-house population of the same background. The presence of at least 1 variant allele and cumulative detection of variant alleles were the only factors significantly and positively associated with HLH severity and refractoriness to treatment. These associations remain true when excluding the most recurrent variant (*PRF1* A91V) from the others. These results suggest that HLHa clinical course and, more specifically, resistance to treatment depend partly on potential genetic predisposition to HLH.

Variants in HLH-related genes have been described in several cohorts of adult patients.[48-51] These studies differ with respect to the racial origin, size of the cohort, age, diagnosis criteria of HLH, genes panel analyzed, methodology to select variants, and clinical end points. The frequency in patients harboring variant alleles ranges from 14%[48] to 43%.[50] But, in all cohorts, including ours, most genetic variations are missense and heterozygous. Some have been found to be expressed as a combination of F-HLH–related gene variants. In this cohort, 43.1% (56 of 130) of patients carried at least 1 variant in the overall population. However, this percentage reached 58% (46 of 80) for severe patients versus 20% (10 of 50) for nonsevere patients. Although this selection of variants may be arguable (minor allelic frequency in reference populations < 5%, CADD phred score ≥ 14.399, and PolyPhen-2 HVAR score ≥ 0.429), their association with the risk of HLH refractoriness and severity supports our filtering strategy.

In favor of genetic involvement in the pathophysiology of the HLHa, some features shared similarities with F-HLH. First, as being found in slight excess when compared with a control cohort adjusted on ethnicity, our potentially pathogenic variants may participate to the onset of HLHa. If so, the mechanism could be that the cumulative effect of potentially minor defects in the degranulation pathway may reach a threshold to develop HLH in context of infection, neoplasia, and/or AID as proposed in recent comprehensive models of HLH pathology.[52,53] Then, the risk of HLH refractoriness or severity correlated with the number of HLH-related gene variants suggests that the addition of variant

alleles in different HLH genes may cooperate to increase the risk of developing severe HLH. Likewise, for 10% of the cohort (12 polyallelic patients of 130 patients), a strict correlation was found between the presence of 2 or more variants and the severity of the disease (severe phenotype). This cumulative effect of variant alleles has previously been observed in murine HLH models. Using mouse models with a combination of heterozygous mutations in *Stx+/−*, *Rab27a+/−*, and *Prf1+/−* genes, the severity of HLH features in mice was found to increase with the number of monoallelic mutations[54] or with the genes involved in combination (ie, *Prf1* or *Rab27a*). In our cohort, 8 of 12 patients with more than 1 allele variant carried at least a variant allele in *PRF1*. Of note, *PRF1/RAB7a* variant allele association was found in 3 patients. As a result, the threshold model can also be translated into "HLH severity," which is likely to increase significantly with the number of variants carried by patients.

In our cohort, 13 patients had at least 1 *STXBP2* mutation and 12 were heterozygous (4 associated with another gene variant). However, only 1 of the 13 patients died within a year following the HLH flare. Therefore, the results observed in our cohort do not confirm the findings of Eloseily et al.[55] Two possible explanations are to be considered for this discrepancy: the age difference between the patients in the 2 cohorts (children vs adults) and the associated pathology with more neoplasia in adult population.

At least, we observed that patients carrying the *PRF1* A91V variant also have an increased risk of severe/refractory HLH. Of the 12 patients carrying the *PRF1* A91V variant, 6 had several variants and (except for 1) were severe cases. In agreement with our findings, the presence of a homozygous or compound heterozygous *PRF1* A91V variant is associated with late-onset HLH or a lethal form of H1N1 HLH.[26,43] However, *in vitro*, its function remains controversial; *PRF1* A91V impairs cytotoxic lymphocyte function[26,42] but is sensitive to temperature. This exogenous factor possibly explains contrasting reports related to the potential pathogenic role of these variants.[56] One limitation of our study is the absence of functional validation of variants. Decrease in natural killer cell cytotoxicity usually reflects a complete or partial loss in HLH-related protein, most of the time, secondary to biallelic mutations as seen in F-HLH. Most of our mutations are heterozygous, and yet mutations acting as a partial "loss of function mutation" have been previously reported in later-onset HLH.[57,58] Thus, we cannot totally eliminate that some of our mutations could act as dominant-negative mutations, impairing the cytotoxic process. However, on the basis of the study of *PRF1* A91V,[56] and also in another study,[51] it will likely be difficult to prove/or disprove their involvement on the basis of currently available *in vitro* functional assays.

Of note, whole exome sequencing studies in patients with HLH have identified variants in genes related to additional pathways unrelated to cytotoxicity, including dysregulation of inflammasome activity, impaired control of virus replication, and primary immunodeficiency.[53,59,60] It is, therefore, tempting to hypothesize that other variants in other genes involved in immune regulation could also play a role, alone or in combination, in influencing the occurrence and severity of HLHa. Further analyses, such as whole exome or genome sequencing, will be needed to test this hypothesis.

One interesting finding of our study was that refractoriness to treatment and disease severity were associated with the presence of variants of HLH genes. Clinicians consider HLH refractoriness a red flag of potential severity for HLHa patients. Therefore, genetic investigations may contribute to a better definition of prognosis and treatment.

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Key messages

- **HLH-related gene variants may contribute to the outcome of HLHa.**
- **Severity or refractoriness to usual treatments may be a clinical signal of the need for genetic investigations in HLHa patients.**

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