Outcome of primary hemophagocytic lymphohistiocytosis: a report on 143 patients from the Italian Registry

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Supplementary methods

Outcome definition

Response to treatment was defined according to the treatment protocol received (HLH-94, HLH-2004, or Euro-HIT). For patients not enrolled in one of these trials, response was assessed at 8 weeks (or before in case of shortened regiments or poor outcome): complete response was defined as a normalization of all the clinical and laboratory HLH criteria (*i.e.*, fever, splenomegaly, hepatomegaly, central nervous system symptoms, absolute neutrophil count, platelet count, hemoglobin, fibrinogen, triglycerides, ferritin, CD25); partial response was defined as resolution of at least two of the aforementioned HLH criteria without appearance of new abnormalities; non response was defined as improvement of less than two HLH criteria or by the development of new abnormalities. Disease reactivation was defined as development of disease manifestations consistent with HLH diagnosis according to the HLH-04 criteria after partial or complete response to treatment.

Genetic studies

Genomic DNA was isolated from peripheral blood samples using BioRobot EZ1 Workstation (Qiagen, Milan, Italy). Peripheral blood samples of patients referred to the Histiocytosis Laboratory of the Meyer Children's Hospital were sequenced by Sanger method or Target Resequencing for the known pHLH-related genes: *PRF1*, *UNC13-D*, *STXBP2*, *STX11*, *RAB27A*, *LYST* (patients with pigment deficiency only), *SH2D1A* and *XIAP/BIRC4* (male patients). For Sanger sequencing, polymerase chain reaction (PCR) was performed on coding exons and adjacent intronic regions. Sequences were performed using the BigDye Terminator Cycle Sequencing Ready Reaction Kit v1.1 or 3.1 (Applied Biosystems, Foster City, California, USA) and 3500 Dx Series Genetic Analyzer CS2 (Thermo Fisher Scientific), then analyzed with the SeqScape software (Applied Biosystems).

Target Resequencing was performed on MySeq platform (Illumina Inc., San Diego, CA). The paired-end reads were aligned to the human hg38 reference genome assembly from Genome Reference Consortium using the Burrows-Wheeler Aligner v0.7.10 (BWA), by reaching a mean read depth of 99X after duplicates removal.

Variant calling was performed using the Unified Genotyper module of the Genome Analysis ToolKit v3.3-0 (GATK). Final variant calling format (VCF) files were filtered and annotated using GoldenHelix VarSeq v.1.3 (Golden Helix, Inc., Bozeman, MT, www.goldenhelix.com). Variants that were called less than 5X, off-target, synonymous, or with minor allele frequency (MAF) in the Exome Aggregation Consortium (ExAC, Cambridge, MA http://exac.broadinstitute.org) were eliminated if not reported in the Human Gene Mutation Database (HGMD). All the variants identified through target resequencing were confirmed by direct sequencing. Variants' nomenclature follows HGVS guidelines (http://www.hgvs.org/mutnomen/).

Table S1. Patients' demographics and clinical characteristics at diagnosis according to the genetic diagnosis

	FHL2 n=47	**FHL3** n=41	**FHL4** n=2	**FHL5** n=15	**XLP1** n=12	**XLP2** n=9	**GS2** n=9	**CHS** n=8	***p* value**
Age (months) – median (IQR)	8 (2-111)	10 (2-88)	75 (NA)	2 (1-8)	54 (34-152)	37 (13-94)	5 (2-11)	14 (2-23)	0.027
Female gender – n (%)	16 (34)	23 (56)	0 (0)	8 (53)	0 (0)	0 (0)	2 (22)	5 (62)	0.002
Geographic origin – n (%) African Caucasian Indian Area Latin American Middle East North African	 3 (6) 39 (83) 2 (4) 1 (2) 0 (0) 2 (4)	 0 (0) 33 (82) 2 (5) 0 (0) 1 (2.5) 4 (10)	 0 (0) 1 (50) 1 (50) 0 (0) 0 (0) 0 (0)	 0 (0) 8 (57) 2 (14) 0 (0) 2 (14) 2 (14)	 0 (0) 11 (92) 0 (0) 1 (8) 0 (0) 0 (0)	 0 (0) 9 (100) 0 (0) 0 (0) 0 (0) 0 (0)	 0 (0) 7 (78) 0 (0) 0 (0) 0 (0) 2 (22)	 0 (0) 5 (62) 0 (0) 1 (12) 0 (0) 2 (25)	0.120
Familial disease – n (%)	8 (18)	14 (34)	0 (0)	5 (33)	4 (33)	1 (11)	1 (11)	4 (50)	0.281
Parental consanguinity – n (%)	7 (15)	8 (20)	0 (0)	5 (33)	1 (8)	0 (0)	4 (44)	5 (62)	0.020
Complete HLH-2004 criteria – n (%)	25 (53)	33 (80)	2 (100)	11 (73)	2 (17)	5 (56)	7 (78)	3 (37)	0.002
Fever – n (%)	41 (87)	37 (90)	2 (100)	12 (80)	8 (67)	11 (100)	9 (100)	5 (62)	0.164
Splenomegaly – n (%)	36 (77)	36 (88)	2 (100)	12 (80)	8 (67)	7 (78)	9 (100)	5 (62)	0.494
Hepatomegaly – n (%)	26 (55)	22 (54)	1 (50)	9 (60)	5 (42)	6 (67)	6 (67)	3 (37)	0.967
CNS involvement[#] – n (%)	15 (32)	13 (32)	0 (0)	3 (20)	6 (50)	2 (22)	5 (56)	5 (62)	0.161
Cytopenia (at least bilinear) – n (%)	29 (62)	33 (80)	2 (100)	11 (67)	2 (17)	4 (44)	8 (89)	4 (50)	0.002
Hemoglobin (g/dL) – median (IQR)	8.2 (7-9.8)	8.1 (6.9-8.7)	9.1 (8.9-9.2)	8.2 (6.8-10.5)	9.9 (9.4-12.4)	9.4 (8.5-11.8)	8.2 (6.9-9)	8.3 (7.8-8.9)	0.061
ANC (cells/uL) – median (IQR)	850 (390-1,790)	530 (260-935)	425 (NA)	1,219 (628-3,335)	2,705 (1,275-4,885)	1,381 (745-4,023)	480 (275-700)	385 (268-938)	0.007
PLTs (cells x10^3/uL) – median (IQR)	52 (29-74)	26 (17-54)	42 (NA)	40 (24-164)	148 (89-243)	152 (82-245)	39 (24-58)	78 (38-214)	0.002
Fibrinogen (mg/dL) – median (IQR)	121 (80-229)	125 (89-239)	167 (NA)	103 (50-140)	103 (95-218)	138 (93-265)	140 (56-233)	180 (103-228)	0.653
Triglycerides (mg/dL) – median (IQR)	243 (172-394)	378 (240-550)	377 (NA)	258 (226-429)	311 (204-497)	196 (188-273)	197 (141-478)	182 (165-215)	0.137
Ferritin (ng/mL) – median (IQR)	3,342 (1,237-12,373)	2,589 (1,227-7,514)	1,958 (NA)	1,480 (1,011-10,885)	2,319 (1,712-11,238)	16,679 (11,935-43,427)	1,842 (864-2,446)	933 (173-1,577)	0.031
ALT (UI/L) – median (IQR)	206 (128-387)	94 (42-159)	61 (NA)	153 (102-398)	105 (64-140)	297 (128-439)	74 (35-99)	81 (41-162)	0.035
AST (UI/L) – median (IQR)	278 (83-473)	103 (59-179)	75 (NA)	159 (142-329)	123 (52-279)	393 (215-881)	109 (44-146)	93 (56-152)	0.106

Bilirubin (mg/dL) – median (IQR)	1.8 (0.7-4.4)	1 (0.6-1.8)	1.3 (0.6-1.8)	0.7 (0.4-1.5)	0.7 (0.4-2.3)	0.9 (0.5-1.3)	0.9 (0.6-2.6)	1.8 (0.7-2.7)	0.761
Infectious trigger – n (%)	8 (17)	9 (22)	1 (50)	3 (20)	7 (58)	7 (78)	4 (44)	3 (37)	0.004

[#] CNS involvement is defined by the presence of at least one of the following: a) neurological symptoms; b) MRI abnormalities; c) CSF abnormalities

IQR: interquartile range; NA: not available; CNS: central nervous system; ANC: absolute neutrophil count; PLTs: platelets; ALT: alanine transaminases; AST: aspartate transaminases; FHL2: familial hemophagocytic lymphohistiocytosis type 2; FHL3: familial hemophagocytic lymphohistiocytosis type 3; FHL4: familial hemophagocytic lymphohistiocytosis type 4; FHL5: familial hemophagocytic lymphohistiocytosis type 5; GS2: Griscelli syndrome type 2; CHS: Chediak-Higashi syndrome; XLP1: X-linked lymphoproliferative disease type 1; XLP2: X-linked lymphoproliferative disease type 2; GS2: Griscelli syndrome type 2; CHS: Chediak-Higashi syndrome; NA: not applicable

Table S2. Impact of treatment on outcome

	Response to first-line treatment			**Status at HSCT**			**Status at last follow-up**		
	Non-response - n (%)	Response - n (%)	*p value*	Dead - n (%)	Alive - n (%)	*p value*	Dead - n (%)	Alive - n (%)	*p value*
First-line treatment									
HLH-94 (n=29)	7 (24)	22 (76)		7 (24)	22 (76)		9 (31)	20 (69)	
HLH-2004 (n=75)	16 (21)	59 (79)	0.847	17 (23)	58 (77)	0.791	27 (36)	48 (64)	0.933
Euro-HIT (n=7)	2 (29)	5 (71)		2 (29)	5 (71)		3 (43)	4 (57)	
Steroids (n=19)	3 (16)	16 (84)		5 (26)	14 (74)		5 (26)	14 (74)	

HSCT: hematopoietic stem cell transplantation

Table S3. Reactivation

First-line treatment before reactivation	Response to first-line treatment	Median time from diagnosis to reactivation (IQR) - *months*	Treatment at reactivation (n)	HSCT, n (%)	Alive at last follow-up, n (%)
Intensive treatment (n=27)	CR/PR (n=23)	5.5 (2.5-9)	HLH-2004 (8) Emapalumab (5) HLH-2004 + ATG (1) HLH-2004 + ruxolitinib (1) HLH-2004 + alemtuzumab (1) None (7)	15 (65)	8 (35)
	NR (n=4)		Steroids (1) Emapalumab (1) HLH-2004 (1) None (1)	2 (50)	0 (0)
Steroids (n=6)	CR/PR (n=6)	13 (3-21)	HLH-94 (2) HLH-2004 (1) Steroids (1) Steroids + sirolimus (1) None (1)	4 (67)	6 (100)

CR: complete response; PR: partial response; NR: non-response; IQR: interquartile range; ATG: antithymocyte globulin; HSCT: hematopoietic stem cell transplantation

Table S4. Patients alive at last follow-up without HSCT

ID	Diagnosis	Mutation	Zygosity	HGMD ID/ Prediction	Functional study*	Comment
687	XLP2	c.1016_1017del, p. Ile339fs	Hemizygous	NR/ Likely Pathogenic	NA	CR to steroids; alive at 27 months of follow-up
708	FHL3	c.1225delC, p.Leu409Ser fs*33; c.2575G>A, p.Ala859Thr	Compound heterozygous	CD113893/ Likely Pathogenic; CM112829/ VUS	Abnormal	Asymptomatic, familial screening (brother died with active HLH); alive at 16 months of follow-up
818	FHL2	c.272C>T, p.Ala91Val	Homozygous	CM022053/ VUS	Abnormal	Active HLH, CR to HLH-94; alive at 27 months of follow-up
888	FHL2	c.698T>C, p.Ile233Thr; c.853_855delAAG, p. Lys285del	Compound heterozygous	NR/VUS; CD011186/ Pathogenic	Abnormal	Asymptomatic; alive at 105 months of follow-up
977	XLP2	c.664C>T, p.Arg222Stop	Hemizygous	CM111971/ Pathogenic	NA	Paucisymptomatic; CR to steroids; alive at 55 months of follow-up
1016	XLP1	c.219T>A, p.His73Gln	Hemizygous	NR/ VUS	NA	Active HLH; CR to HLH-2004; alive at 85 months of follow-up
1053	FHL2	c.272C>T, p.Ala91Val	Homozygous	CM022053/ VUS	Abnormal	Active HLH; CR to steroids; alive at 86 months of follow-up
1165	FHL3	c.321+2 T>G; c.2346_2349 del GGAG, p. Arg782Ser fs*12	Compound heterozygous	NR/ Likely Pathogenic; CD060676/ Pathogenic	Abnormal	PR to HLH-2004; alive at 35 months of follow-up
1211	XLP2	c.641A>G, p.Asp214Gly	Hemizygous	NR; VUS	Abnormal	Active HLH; CR to steroids; alive at 48 months of follow-up
1338	CHS	c.10669G>T, p. Val3557Leu and other 6 missense variants	Compound Heterozygous	NR/ VUS; Other 6: NR/ Benign	NA	Albinism, CNS symptoms; alive at 14 months of follow-up

1353	FHL3	c.1407G>A, p.Trp469Stop; c.2346_2349 del GGAG, p. Arg782Ser fs*12	Compound Heterozygous	NR/ Likely Pathogenic; CD060676/ Pathogenic	Abnormal	Active HLH; PR to HLH-2004; alive at 13 months of follow-up
1410	FHL2	c.272C>T, p.Ala91Val	Homozygous	CM022053/ VUS	Abnormal	Active HLH; CR to HLH-94; alive at 51 months of follow-up
1433	XLP2	c.331C>T, p.Gln111Stop	Hemizygous	CM2130297/ Likely Pathogenic	NA	Paucisymptomatic; PR to HLH-94; alive at 13 months of follow-up
1513	FHL2	c.272C>T, p.Ala91Val; c.695G>A, p.Arg232His	Compound Heterozygous	CM022053/ VUS; CM021668/ Likely Pathogenic	Abnormal	Asymptomatic, familial screening (first degree relative with active HLH); alive at 16 months of follow-up
1522	FHL5	c.1247-1G>C; c.325_325+3delAGTG, p.Thr109fs	Compound Heterozygous	CS096295/ Pathogenic; NR/Likely Pathogenic	Abnormal	Asymptomatic; alive at 34 months of follow-up

*Functional study refers to perforin expression evaluation for FHL-2 and to degranulation assay for the other genetic forms

NR: not reported; NA: not available; VUS: variant of uncertain significance

Table S5. Causes of death

Category	**Description (number of events)**	**Median time from diagnosis to death (IQR) -** *months*
Disease progression (n=32)	Unspecified/MOF (24)	1 (1-3)
	Liver failure (3)	
	CNS progression (2)	
	Post-HSCT reactivation (3)	
HSCT related mortality (n=11)	HSCT toxicity (7)	11 (7-25)
	Infection (4)	
Infection (n=5)	Sepsis (4)	1 (0.5-6.5)
	Pneumonia (1)	
Sequelae (n=2)	Severe CNS disability	23.5 (NA)

HSCT: hematopoietic stem cell transplantation; MOF: multi-organ failure; CNS: central nervous system; IQR: interquartile range; NA: not available

Figure S1. Genetic diagnoses per age group

FHL2: familial hemophagocytic lymphohistiocytosis type 2; FHL3: familial hemophagocytic lymphohistiocytosis type 3; FHL4: familial hemophagocytic lymphohistiocytosis type 4; FHL5: familial hemophagocytic lymphohistiocytosis type 5; GS2: Griscelli syndrome type 2; CHS: Chediak-Higashi syndrome; XLP1: X-linked lymphoproliferative disease type 1; XLP2: X-linked lymphoproliferative disease type 2; CHS: Chediak-Higashi syndrome; GS2: Griscelli syndrome type 2

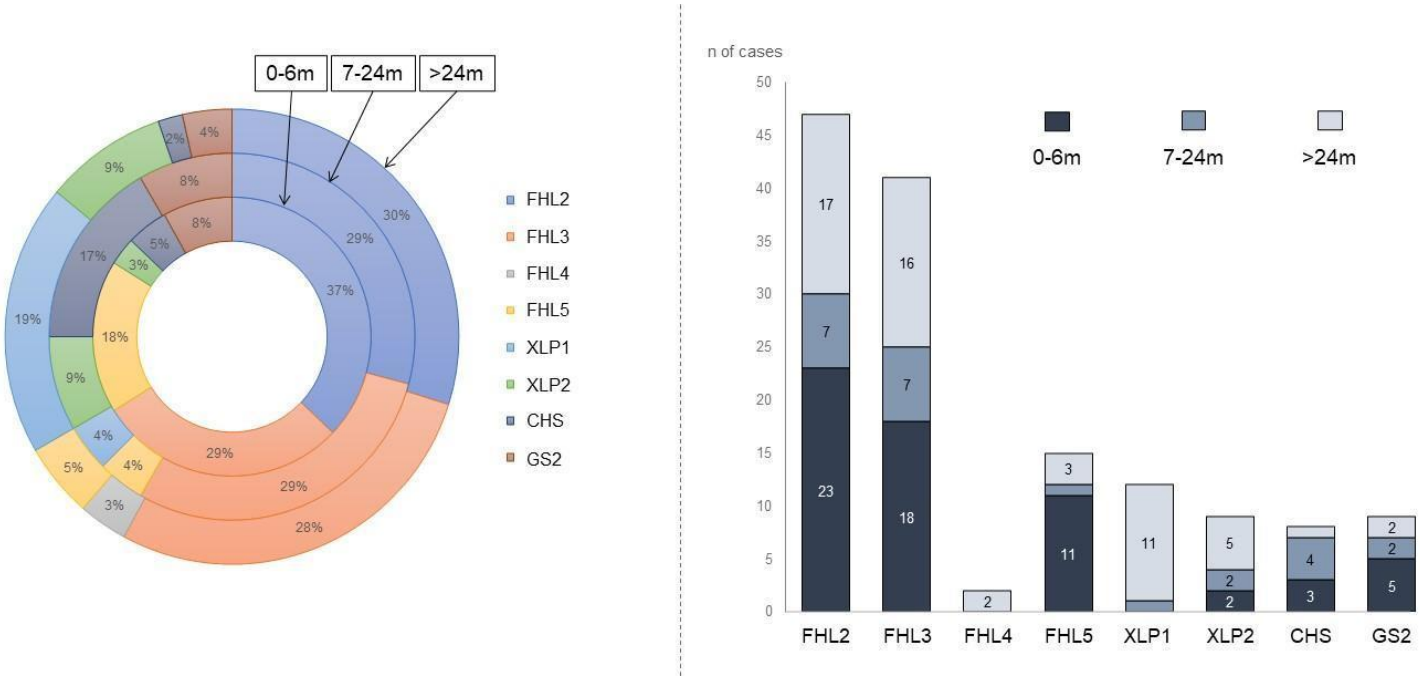


Figure S2. Variation of baseline features according to the genetic diagnosis

FHL2: familial hemophagocytic lymphohistiocytosis type 2; FHL3: familial hemophagocytic lymphohistiocytosis type 3; FHL4: familial hemophagocytic lymphohistiocytosis type 4; FHL5: familial hemophagocytic lymphohistiocytosis type 5; GS2: Griscelli syndrome type 2; CHS: Chediak-Higashi syndrome; XLP1: X-linked lymphoproliferative disease type 1; XLP2: X-linked lymphoproliferative disease type 2; GS2: Griscelli syndrome type 2; CHS: Chediak-Higashi syndrome; AST: aspartate aminotransferase; ALT: alanine aminotransferase

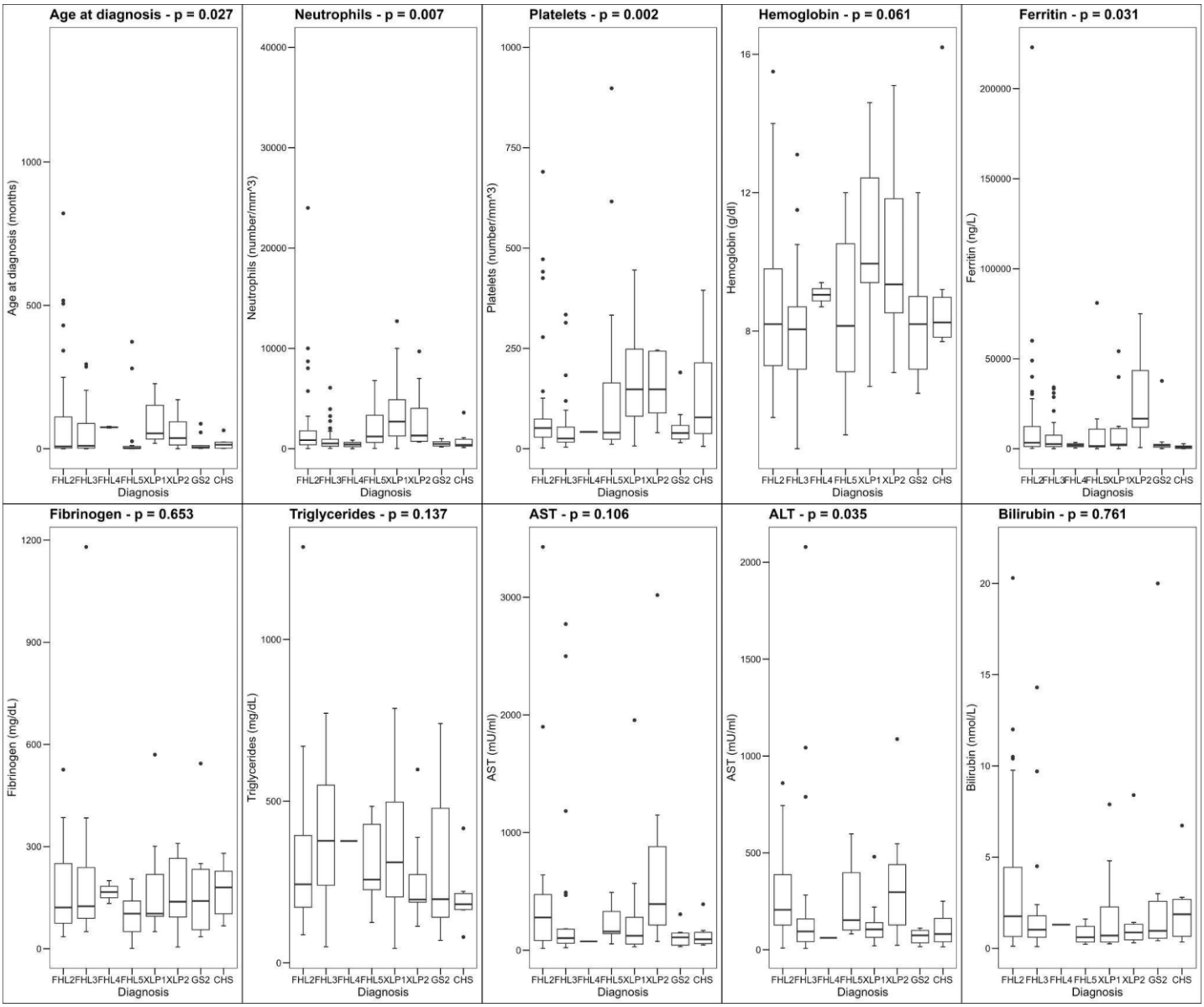


Figure S3. Survival analysis according to the genetic diagnoses

FHL2: familial hemophagocytic lymphohistiocytosis type 2; FHL3: familial hemophagocytic lymphohistiocytosis type 3; FHL4: familial hemophagocytic lymphohistiocytosis type 4; FHL5: familial hemophagocytic lymphohistiocytosis type 5; GS2: Griscelli syndrome type 2; CHS: Chediak-Higashi syndrome; XLP1: X-linked lymphoproliferative disease type 1; XLP2: X-linked lymphoproliferative disease type 2; GS2: Griscelli syndrome type 2; CHS: Chediak-Higashi syndrome; HSCT: hematopoietic stem cell transplantation

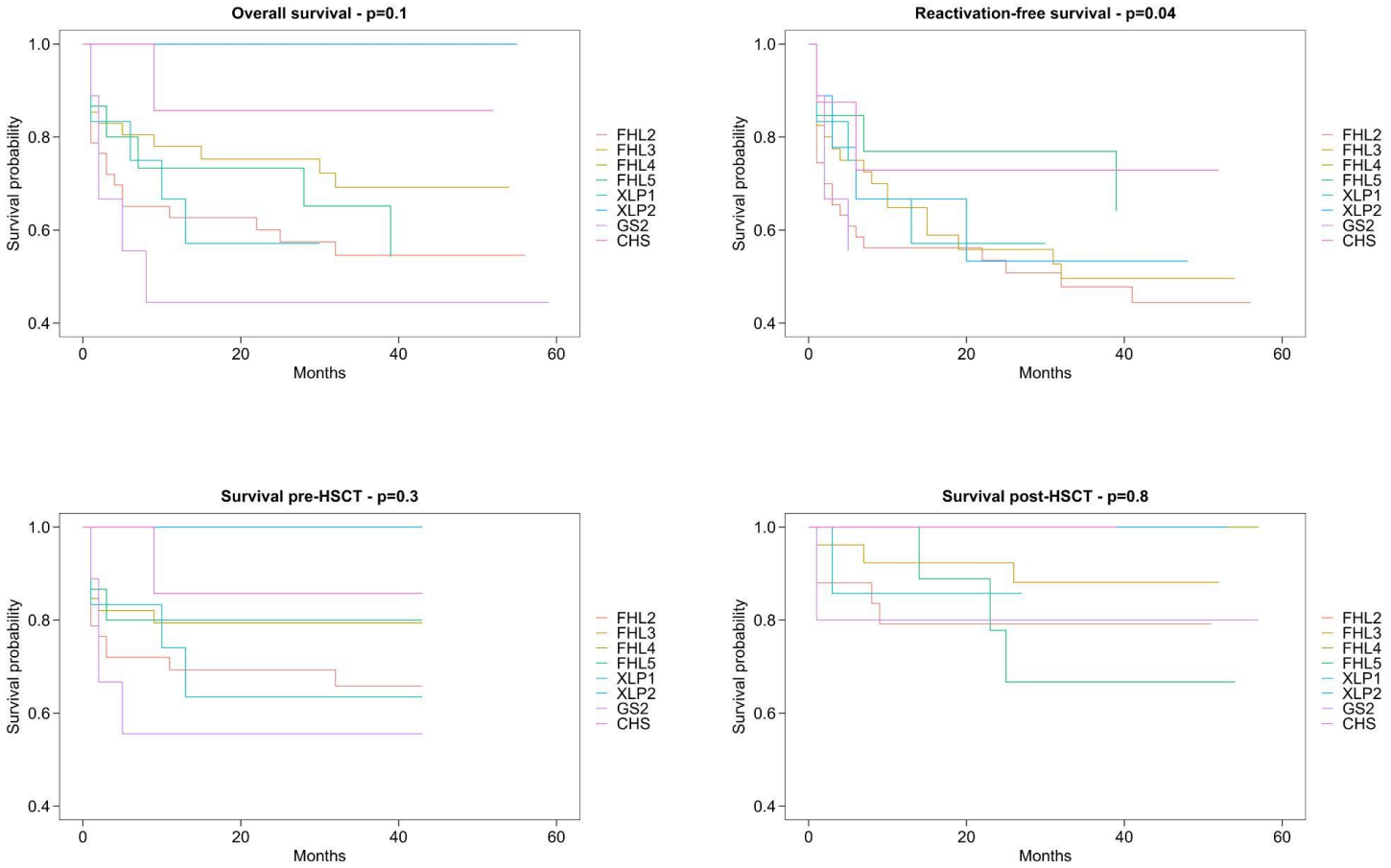
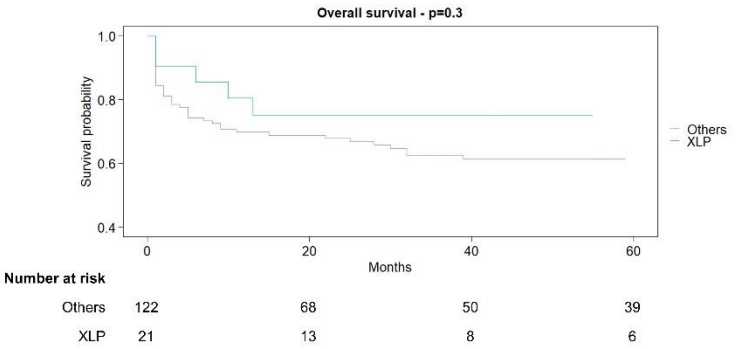
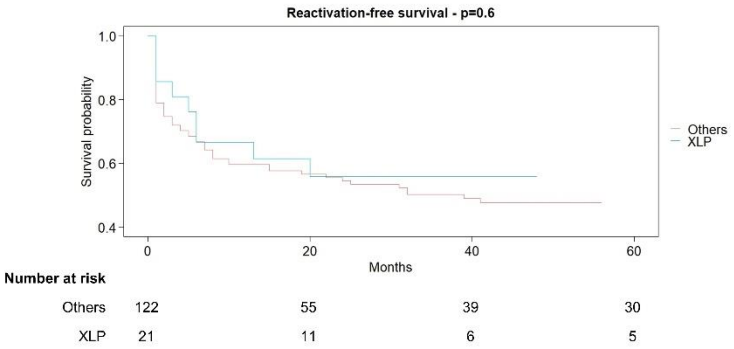
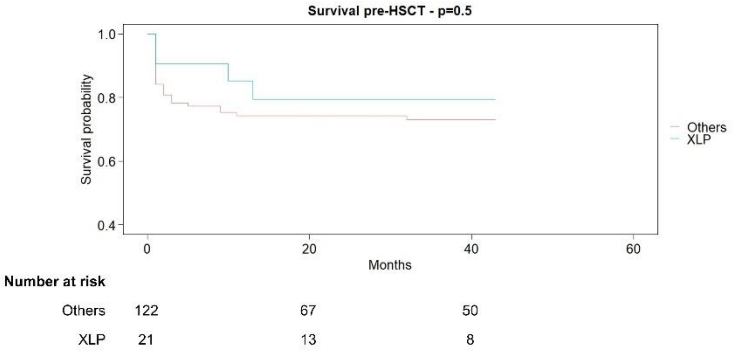


Figure S4. Survival analysis comparing patients with FHL and pigmentary disorders, and patients with XLP1-2

FHL: familial hemophagocytic lymphohistiocytosis; XLP: X-linked lymphoproliferative disease; HSCT: hematopoietic stem cell transplantation







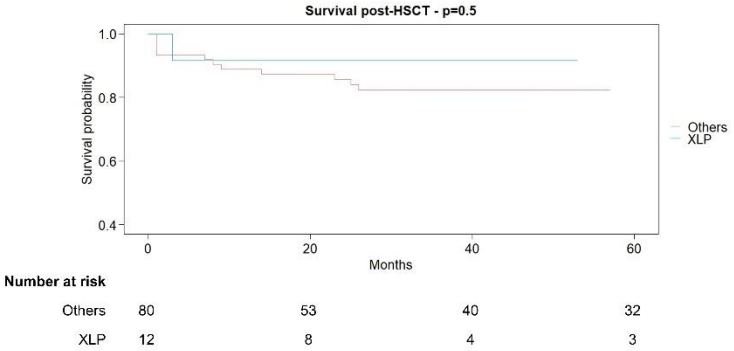


Figure S5. Survival analysis comparing the first (2007-2014) and the second (2015-2022) study period

HSCT: hematopoietic stem cell transplantation