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REPORT

A form of inherited hyperferritinemia associated with bi-allelic pathogenic variants of *STAB1*

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Summary

Hyperferritinemia is a frequent finding in several conditions, both genetic and acquired. We previously studied eleven healthy subjects from eight different families presenting with unexplained hyperferritinemia. Their findings suggested the existence of an autosomal-recessive disorder. We carried out whole-exome sequencing to detect the genetic cause of hyperferritinemia. Immunohistochemistry and flow cytometry assays were performed on liver biopsies and monocyte-macrophages to confirm the pathogenic role of the identified candidate variants. Through a combined approach of whole-exome sequencing and homozygosity mapping, we found bi-allelic *STAB1* variants in ten subjects from seven families. *STAB1* encodes the multifunctional scavenger receptor stabilin-1. Immunohistochemistry and flow cytometry analyses showed absent or markedly reduced stabilin-1 in liver samples, monocytes, and monocyte-derived macrophages. Our findings show a strong association between otherwise unexplained hyperferritinemia and bi-allelic *STAB1* mutations suggesting the existence of another genetic cause of hyperferritinemia without iron overload and an unexpected function of stabilin-1 in ferritin metabolism.

Introduction

Hyperferritinemia is a frequent finding in clinical practice and often requires an extensive diagnostic workup. A large spectrum of conditions, both genetic and acquired, associated or not with iron overload, displays high serum ferritin.[1–3] The diagnostic strategy to reveal the cause of hyperferritinemia includes family and personal medical history, biochemical and genetic tests, and evaluation of liver iron by direct (biopsy) or indirect (quantitative magnetic resonance) methods.[1] Despite this complex and time-consuming approach, often the precise etiology remains elusive.

Ferritin expression in mammals is regulated by iron through a well-characterized mechanism of coordinated cytosolic post-transcriptional regulation.[4] In addition to iron, ferritin synthesis is regulated by cytokines during development, cellular differentiation, proliferation, and inflammation.[5] In mammals, a small amount of ferritin (normally 0.025% of the total body ferritin)[6] is present in a secreted form in serum. It mostly consists of variably glycosylated L-ferritin and trace amounts of H-ferritin.[7,8] Different from cytosolic ferritin, extracellular ferritin is relatively poor in iron.[7,8] Serum ferritin measurement has become a routine laboratory test to indirectly evaluate iron stores, although it is known that many additional factors, including inflammation, infection, liver diseases, and dietary and metabolic abnormalities—all of which may elevate serum ferritin—complicate its interpretation.[1,2,9] Despite this long history of clinical use, fundamental aspects of the biology of serum ferritin are still unclear. For example, tissue of origin, secretory pathway, receptor interactions, clearance, and functions remain topics of active debate.[10–12]

Stabilin receptors belong to class H scavenger receptors that consists of two members, stabilin-1 (also known as Clever-1 and FEEL-1) and stabilin-2.[13,14] The stabilins are enigmatic proteins whose physiological functions are still not entirely understood.[15] They comprise a large extracellular N terminus of multiple epidermal growth factor (EGF)/EGF-like domains, seven fasciclin-1 domains, an X-link domain, and a short intracellular C-terminal domain, linked by a transmembrane region.[14] Their extracellular domains share 55% similar homology, but their short intracellular domains are highly diverse, which results in differential abundance and function in different tissues and cells.[15,16] More specifically, stabilin-1 is primarily expressed on human monocytes, immunosuppressive macrophage populations, lymphatic endothelial cells, and sinusoidal endothelial cells of the liver, spleen, adrenal cortex, and bone marrow, and is involved in scavenging, angiogenesis, and cell adhesion.[14,16,17] As a scavenger receptor, stabilin-1 is known to bind and endocytose a wide range of ligands and, therefore, plays an important role in tissue homoeostasis and remodeling, and is involved in receptor-mediated endocytosis, intracellular sorting, and recycling.

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Table 1. Iron indices at diagnosis in the 11 subjects with hyperferritinemia

Subjects		Gender	Age (yrs)	Follow-up (months)	Alcohol intake (g/day)	Hb (g/dL)	TSAT (%)	s-Ferr (μg/L)	MR-Liver T2* (ms)	MR-LIC (mg/g)
Family A	#1	M	21	194	<5	14.9	41	1,313	15.2	1.67
Family B	#2	M	53	184	0	14.5	33	4,654	10.6	2.42
	#3	M	49	184	<5	15.6	33	2,947	21.6	1.16
Family C	#4	F	32	125	0	14.0	34	987	23.8	1.05
	#5	F	34	82	0	13.1	28	365	28.6	0.87
Family D	#6	M	30	82	<5	13.1	20	1,970	15.2	1.67
	#7	M	37	55	<5	17.0	27	2,109	14.4	1.76
Family E	#8	M	60	120	<5	13.1	26	3,226	14.8	1.72
Family F	#9	M	56	62	<5	15.9	40	1,645	17.2	1.47
Family G	#10	M	61	64	<5	16.7	26.5	2,903	22.0	1.14
Family H	#11	M	63	76	0	13.6	38	1,154	14.8	1.72

Abbreviations: TSAT, transferrin saturation; s-Ferr, serum ferritin; MR, magnetic resonance; LIC, liver iron concentration. Normal ranges: Hb 14–18 g/dL in men and 12–14 g/dL in women; transferrin saturation (TSAT) 16%–45%; serum ferritin (s-Ferr) 30–400 μg/dL in men and 13–150 μg/dL in women; MR T2* 14–32 ms; the MR-LIC ranges derived from MR T2* according to Galimberti et al.[19] are 0.7–1.8 mg/g.

In 2017 we described several Italian subjects with unexplained isolated hyperferritinemia.[18] Four probands had affected siblings but no affected parents or offspring, suggesting the existence of an inherited form of hyperferritinemia without iron overload manifesting as an autosomal-recessive trait. We are now able to reveal the presence of bi-allelic pathogenic variants affecting the multifunctional scavenger receptor stabilin-1 gene (*STAB1* [MIM: 608560]) in most of those probands and affected siblings, suggesting that stabilin-1 might have an important role in the regulation of serum ferritin levels in humans.

Material and methods

Subjects

Of the twelve subjects originally reported by Ravasi et al.,[18] eleven were available for this study and underwent whole-exome sequencing (WES). They are all Italians and belong to eight different families presenting unexplained hyperferritinemia without iron overload. All of them were in good health and had no dysmorphologies, psycho-motor development abnormalities, hearing or vision disorders, or other pathologies. None of them had a history of high alcohol intake nor of any of the known inherited or acquired causes of hyperferritinemia. Their demographic and clinical data have been previously reported[18] and their iron data are summarized in Table 1. These subjects showed levels of serum ferritin markedly higher than expected according to age and gender, ranging from 365 ng/mL to 4,654 μg/L. Transferrin saturation (TSAT) was normal (range 20%–41%), as well as the other laboratory parameters (blood count, liver function tests, and metabolic and inflammatory indices). Quantification of liver iron by magnetic resonance showed normal values. Family history collection revealed distant consanguinity in the probands' parents of family A (#1) and B (#2 and #3).

All probands and available relatives gave their written informed consent for genetic testing and research use according to the Institutional Review Board of Fondazione IRCCS - San Gerardo dei Tintori (protocol code Gen-CI-001A and GEN-CI-012 approved on 12 October 2018) and for the study "HyFerr" (protocol 2973 approved by the Institutional Ethical Committee on 24 March 2022; registered number NCT05659017 at ClinicalTrials.gov). All the hyperferritinemic subjects were negative for causal mutations in hemochromatosis-related genes (*HFE* [MIM: 613609], *HAMP* [MIM: 606464], *HJV* [MIM: 608374], *SLC40A1* [MIM: 604653], *TFR2* [MIM: 604720]) and in L-ferritin gene (*FTL* [MIM: 134790]) causing hyperferritinemia with or without cataract syndrome (MIM: 600886).

Genetic methods

WES was carried on using the Nextera Rapid Capture Exome Library kit (Illumina) on the Illumina NextSeq 550 System. Reads alignment and variant calling/annotation were performed using BWA-MEM algorithm and Picard-GATK4 tools (Broad Institute). Homozygosity mapping was performed starting from WES data using the AutoMap online tool (https://automap.iob.ch/) (parameters: DP = 8, percaltlow = 0.25, percalthigh = 0.75, binomial = 0.000001, maxgap = 10, window = 7, windowthres = 5, minsize = 2, minvar = 25, minperc = 88, chrX = no, extend = 1).[20] WES variants were prioritized using the following criteria: allele frequency (MAF) $\leq$ 0.001, quality score (QS) $\geq$ 30, protein impact, *in silico* prediction of pathogenicity (CADD, DANN, PolyPhen2, SIFT-PROVEAN, MutationTaster),[21–25] homozygous or compound heterozygous status, and genotype identity in affected siblings (when applicable). The candidate variants were validated by Sanger sequencing in the probands and available parents.

Immunohistochemistry

Three subjects (#9, #2, and #3) underwent liver biopsy for diagnostic purposes in the past. Blank samples were obtained from Formalin-Fixed Paraffin-Embedded (FFPE) specimens. We collected control liver samples from subjects with hypertransaminasemia whose histology was normal (kindly provided by the hepatology outpatient clinic of the Fondazione IRCCS - San Gerardo

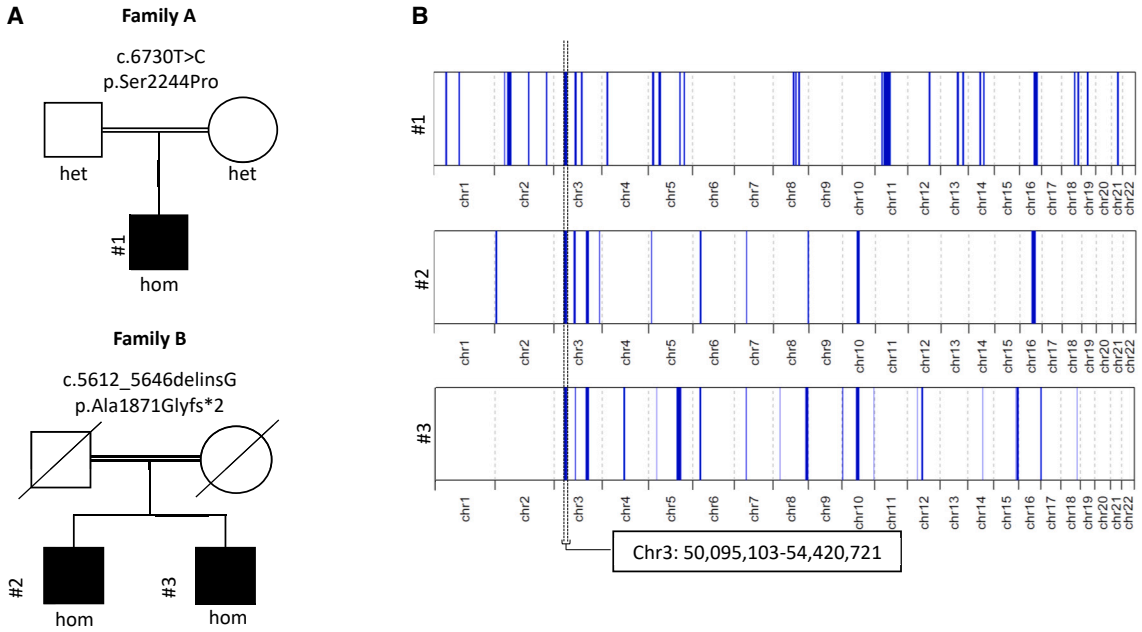

Figure 1. Pedigrees and homozygosity mapping analyses of the two families with history of consanguinity
(A) Pedigrees of the two families with history of distant consanguinity carrying the candidate *STAB1* variants (GenBank: NM_015136). Black symbols denote affected status (hom, homozygous; het, heterozygous).
(B) Homozygosity mapping analysis in subjects #1 (family A), #2 and #3 (family B), performed with AutoMap online tool (https://automap.iob.ch/), revealed a single chromosomal region of homozygosity overlap on chromosome 3 (hg19, chr3: 50,095,103–54,420,721 bp), where *STAB1* is localized (chr3: 52,529,354–52,558,511).

dei Tintori). FFPE 4–5 μm slices were stained for stabilin-1 using Ventana Benchmark Ultra (Roche Diagnostics) with an anti-stabilin-1 primary antibody (clone 4G9, sc-293254, Santa Cruz) at 1:100 dilution combined with UltraView Universal DAB Detection Kit (Roche Diagnostics). Anti-Vascular adhesion protein 1 (VAP1) antibodies were used to check for liver integrity of the analyzed samples (polyclonal rabbit anti-serum against human VAP1, produced in-house and used 1:5,000).

Flow cytometry

Mononuclear cells were isolated by Ficoll gradient centrifugation (GE Healthcare, Amersham Biosciences Europe GmbH) from 40 mL of blood of the affected individuals (#2, #3, #1, and #10) and healthy control subjects, and human monocytes were isolated from peripheral blood mononuclear cells by magnetic enrichment using $CD14^+$ microbeads according to the manufacturer's instructions (Miltenyi Bergisch Gladbach). Monocytes were used as such or differentiated (subjects #2 and #1) into macrophages and M2 polarized as previously described.[26] For flow cytometry, cells were stained with anti-stabilin-1 (clone 9–11, *InVivo* Biotech Hennigsdorf) conjugated with Alexa Fluor 647 in-house,[27] anti-CD14-Pacific Blue (558121, BD), or isotype-matched irrelevant antibody. 7-AAD was used as a viability dye (Invitrogen, 00-6993-50). Samples were acquired on the LSRFortessa (BD) and analyzed with FlowJo v.10.8.1 (BD).

Results

Genetic study

In the hypothesis of a recessive phenotype, we searched for rare homozygous or compound heterozygous variants in WES data. Based on the iron phenotype of the subjects, we did not expect and did not find pathogenic variants in the hemochromatosis genes or genes associated with hereditary iron overload. We also confirmed the negative findings obtained with Sanger sequencing in L-ferritin and ferroportin genes.[18] The single individual of family A (#1) and the two siblings of family B (#2 and #3) showed a higher homozygosity rate than control subjects in line with the known distant consanguinity of their parents (below 5 generations from probands). Homozygosity mapping showed a single chromosomal region of homozygosity overlap in the three subjects on chromosome 3 (hg19, chr3: 50,095,103–54,420,721 bp) (Figure 1). Looking for rare non-synonymous variants in this locus, we found two homozygous variants in the *STAB1* gene (GenBank: NM_015136): a missense variant (c.6730T>C [p.Ser2244Pro]) in subject #1 and a deletion/insertion variation (c.5612_5646delinsG) leading to a premature stop codon (p.Ala1871Glyfs*2) in subjects #2 and #3. Interestingly, gnomAD data suggest that *STAB1* is mildly-to-moderately intolerant to missense and loss-of-function variants (o/e = 0.92 and 0.68, respectively). In addition, a recent meta-analysis of genome-wide association studies (GWASs) found likely pathogenic variants *STAB1* variants associated with ferritin levels.[28] Therefore, *STAB1* was prioritized as the candidate etiological gene and was analyzed in the remaining probands with unexplained hyperferritinemia. Remarkably, we found *STAB1* variants in seven of the remaining eight subjects with hyperferritinemia. In details, six carried rare compound heterozygous variants in *STAB1*: c.1042G>A (p.Glu348Lys)

Table 2. Genetic characterization of the identified *STAB1* variants

Subjects	Variants (cDNA)	Variants (aa)	Allelic status	*In silico* predictions	Mammalian conservation	rs number (dbSNP)	MAF (gnomAD_exomes)
#1	c.6730T>C	p.Ser2244Pro	homozygous	deleterious (5/5)	yes	rs141939118	0.000028
#2 and #3	c.5612_5646delinsG	p.Ala1871Glyfs*2	homozygous	deleterious (2/2)	N/A	N/A	N/A
#4 and #5	c.1042G>A	p.Glu348Lys	heterozygous	deleterious (4/5)	yes	rs754318051	0.000005
	c.7328G>A	p.Trp2443*	heterozygous	deleterious (3/3)	N/A	rs748728975	0.000004
#6 and #7	c.2169_2177del	p.Phe724_Gly726del	heterozygous	likely deleterious (1/2)	yes	N/A	N/A
	c.3364dup	p.Arg1122Profs*37	heterozygous	deleterious (2/2)	N/A	rs563085224	0.000064
#8	c.6730T>C	p.Ser2244Pro	heterozygous	deleterious (5/5)	yes	rs141939118	0.000028
	c.7016A>G	p.Tyr2339Cys	heterozygous	deleterious (5/5)	yes	rs1484029620	0.000014
#9	c.358T>A	p.Cys120Ser	heterozygous	deleterious (5/5)	yes	rs778572255	0.000004
	c.2352C>T	p.Cys784=	heterozygous	not deleterious (0/2)	no	rs898828640	0.000012
#10	c.6730T>C	p.Ser2244Pro	homozygous	deleterious (5/5)	yes	rs141939118	0.000028

Abbreviations: AA, amino acid; MAF, minor allele frequency; N/A, not applicable. The following tools have been used for the *in silico* predictions: CADD, DANN, PolyPhen2, SIFT-PROVEAN, MutationTaster, SpliceAI, VarSEAK, Human Splicing Finder (when applicable).

and c.7328G>A (p.Trp2443*) in subjects #4 and #5 (family C), c.2169_2177del (p.Phe724_Gly726del) and c.3364dup (p.Arg1122Profs*37) in subjects #6 and #7 (family D), c.6730T>C (p.Ser2244Pro) and c.7016A>G (p.Tyr2339Cys) in subject #8 (family E), and c.358T>A (p.Cys120Ser) and the rare synonymous variant c.2352C>T (p.Cys784=) in subject #9. Last, proband #10 was homozygous for the c.6730T>C (p.Ser2244Pro) variant, which was also observed in proband 1 in homozygosity and in proband #3 in compound heterozygosity. Interestingly, the three persons carrying this variant (#1, #8, and #10) originated from a restricted geographical area in the south of the Apulia region suggesting a founder effect. All the variants were confirmed by Sanger sequencing. The available parents, who presented normal serum ferritin, were heterozygous carriers of the *STAB1* variants, demonstrating that *STAB1* variants were in *trans* on different chromosomes in all probands. Table 2 summarizes the identified *STAB1* variants and Figures S1 and S2 show the family pedigrees and *STAB1* variants visualization in the .bam files of the affected subjects, respectively.

Immunohistochemistry and flow cytometry

To understand how the genetic variants affected stabilin-1 protein levels, we stained liver biopsy samples from hyperferritinemic subjects #2, #3, and #9 and control subjects with an anti-stabilin-1 antibody. In the liver, stabilin-1 is known to be expressed by liver sinusoidal endothelial cells but not by Kupffer cells.[29] Samples from #2, #3, and #9 subjects showed no immunoreactivity with anti-stabilin-1 compared to control liver where high signal was detected in the liver sinusoids (Figure 2). All the samples stained normally with anti-vascular adhesion protein 1 (VAP-1) antibody used as control staining confirming the integrity of the samples (Figure S3). Thus, the staining results suggest that the identified stabilin-1 genetic variants lead to complete loss of the expressed protein in the liver.

To validate this finding on a more accessible cell population, we next investigated stabilin-1 expression levels on peripheral monocytes (#2, #1, #3, and #10) and monocyte-derived macrophages obtained from individuals #2 and #1. Consistently, we observed very little expression of stabilin-1 on $CD14^+$ monocytes and macrophages compared to control subjects (Figure 3).

Discussion

There are several lines of evidence supporting the pathogenic role of the identified *STAB1* variants and their relations with hyperferritinemia in the subjects studied. First, the co-segregation of bi-allelic *STAB1* variants with hyperferritinemia in three unrelated families (#2 and #3, #4 and #5, #6 and #7) is a major proof of pathogenicity. Second, the nonsense variants (p.Ala1871Glyfs*2, p.Trp2443*, p.Arg1122Profs*37) are highly deleterious as they are expected to cause a premature stop codon leading to a truncated stabilin-1 protein. Third, the identified single-nucleotide variants and small indels are extremely rare (identified only in the heterozygous status in the population database gnomAD)[30] affecting amino acids highly conserved across mammalian species and predicted to be deleterious by the majority of *in silico* tools, except for the p.= variant carried by subject #9 (Table 2). This variant deserves separate consideration since it is synonymous, it does not affect a conserved nucleotide, and *in silico* tools predict it to be benign. A splicing disruption effect can be hypothesized as shown in other inherited disorders.[31] Indeed, Human Splicing Finder suggests a significant alteration of ESE/ESS motifs ratio for the variant p.Cys784=, although other *in silico* prediction tools do not support

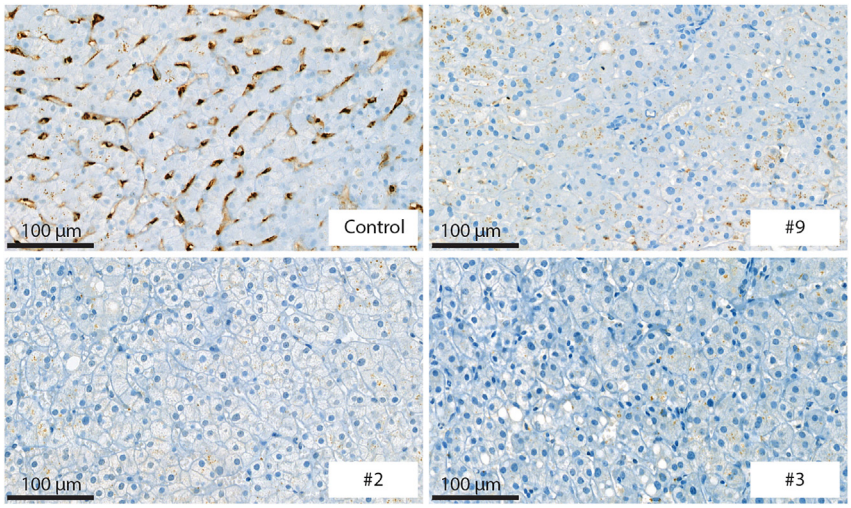

Figure 2. Anti-stabilin-1 staining of liver biopsy samples from a healthy control and subjects #9, #2, and #3 (40×)

this hypothesis (i.e., SpliceAI, VarSEAK). Unfortunately, RNA from subject #9 was not available for *STAB1* transcript analysis. In the hypothesis of other pathogenic variants in the non-coding regions of STAB1, his raw whole exome-sequencing data were carefully analyzed for rare variants at the exon-intron boundaries up to 50 bases away from the splice site, but no additional rare intronic variants were identified. However, liver immunohistochemistry (Figure 2) indicates that proband #9 is unable to express stabilin-1 protein as observed in subjects #2 and #3 carrying nonsense mutations, indicating that he carries a deleterious *STAB1* genotype. Fourth, the analysis of in-house exomes of 500 subjects without hyperferritinemia (Italian individuals with neurological diagnoses) did not reveal any carrier of these specific variants or rare bi-allelic *STAB1* variations (data not shown). Fifth, a recent GWAS meta-analysis found an association between four uncorrelated rare *STAB1* variants (p.Glu117*, p.Gly189Ser, p.Glu527Lys, and p.Ser1089Gly) and increased serum ferritin, and in line with our observations, those *STAB1* variants did not associate with increased iron levels and transferrin saturation.[28] Sixth, immunohistochemistry studies displayed a striking reduction of stabilin-1 protein in hepatic sinusoidal cells of *STAB1* mutation carriers compared to control subjects. Seventh, blood monocytes and macrophages from hyperferritinemic subjects displayed a marked decrease of stabilin-1 on cell surfaces.

Stabilin-1 is a very versatile molecule acting as a scavenger receptor for clearing degradation products from the circulation.[14] Moreover, unexpectedly for scavenger receptors, it is involved in receptor-mediated endocytosis, intracellular sorting, and recycling.[32] Due to the multifaceted function of stabilin-1, different hypotheses can be drawn to explain our findings. First, stabilin-1 may have a role as a scavenger receptor for serum ferritin at the surface of liver sinusoidal endothelial cells and/or macrophages. There is general agreement that cells uptake ferritin through two routes: direct uptake by ferritin receptors and indirect uptake by ferritin-binding proteins.[33] Ferritin receptors have been previously identified in different cell lines. TIM2 has no human ortholog and human TFR1 is a cell surface receptor for H ferritin.[34,35] SCARA 5, which belongs to scavenger receptor class A, mediates the uptake of ferritin iron in specific cell types in the developing kidney in mice,[36] and SCARA5-transfected cells can bind or internalize human L-ferritin and H-ferritin.[37] Stabilin-1, which is able to endocytose ligands such as low-density lipoproteins, Gram-positive and Gram-negative bacteria, and advanced glycosylation end products,[14,38] might be another scavenger receptor for ferritin in other cell types. Second, stabilin-1 might control ferritin metabolism through more complex mechanisms that concern its role in the regulation of the macrophage secretome.[32] Stabilin-1 shuttles between endosomal compartment and biosynthetic compartment and transports newly synthesized stabilin-interacting proteins to the lysosomal secretory pathway. Interestingly, it has been previously proposed that serum ferritin is secreted through the non-classical lysosomal secretory pathway in mice, specifically through secretory lysosomes.[10] This is supported by the recent findings showing that specific defects of proteins involved in early or later stages of endo-lysosomal trafficking have opposite effects on ferritin secretion.[39] Both hypotheses are in agreement with what we have previously observed in these subjects who showed elevated serum ferritin levels in the face of normal ferritin concentrations in lympho-monocytes suggesting altered ferritin secretion or clearance.[18]

In conclusion, our findings show a strong association between otherwise unexplained hyperferritinemia and bi-allelic *STAB1* mutations suggesting the existence of another genetic cause of hyperferritinemia without iron overload and an unexpected function of stabilin-1 in ferritin metabolism. The identification of *STAB1* bi-allelic mutations in other persons with unexplained hyperferritinemia is needed to confirm our findings, thus supporting *STAB1* sequencing as a new tool in the differential diagnosis of hyperferritinemia.

Although the association between *STAB1* mutations and hyperferritinemia is strongly supported by the data, the underlying mechanisms linking stabilin-1 to ferritin remain to be defined and requires further studies to be clarified. At present, there is no evidence that *STAB1* mutations can lead to clinical manifestations other than hyperferritinemia as all the persons reported here were still in good health after 112 ± 53 months of follow-up. This is in line with the findings that stabilin-1 full or

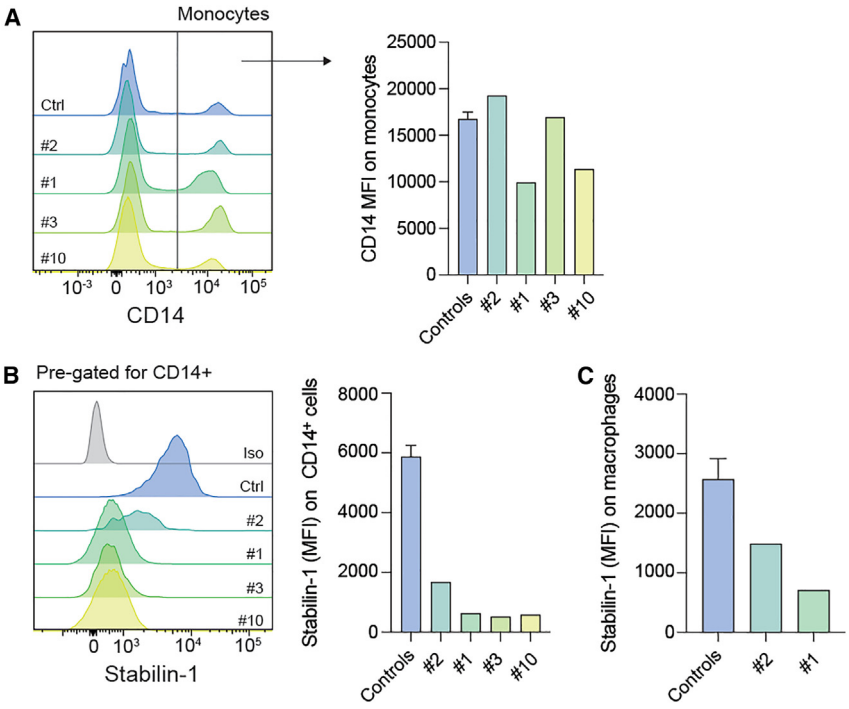

Figure 3. Flow cytometric analysis of CD14 expression in cells of affected individuals and controls

Flow cytometric analysis of CD14 expression (MFI, median fluorescence intensity) on peripheral blood monocytes (A), cell-surface stabilin-1 on $CD14^+$ monocytes in control subjects (n = 3) and in subjects #2, #1, #3, and #10 (B), and on monocyte-derived macrophages from control subjects (n = 3) and subjects #2 and #1 (C). Gray histogram in (B) shows isotype control (iso) staining for stabilin-1.

conditionally knockout mice show normal phenotypes.[40] However, later manifestations cannot be excluded considering the role of stabilin-1 in controlling inflammatory response.[27]

Data and code availability

The article contains identifiers or accession numbers linking to information on known genes and diseases deposited in public databases (OMIM, GenBank RefSeq). The sequencing files supporting the current study have not been deposited in a public repository because the subjects participating in this study did not give consent to upload their genomic data. However, WES data are available from the corresponding author upon reasonable request.

Supplemental information

Supplemental information can be found online at https://doi.org/10.1016/j.ajhg.2023.07.004.

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Author contributions

S.P. and A.P. designed research; E.M., S.P., M.H., M.V., F.B., and A.D.F. performed experiments; E.M., S.P., R.M., S.M., A.D.F., and A.P. collected and/or analyzed data; E.M., S.P., M.H., S.M., A.D.F., and A.P. wrote the manuscript; and all authors revised the manuscript.

Declaration of interests

The authors declare no competing financial interests.

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