VoxSanguinis
The International Journal of Transfusion Medicine
ISBT International Society of Blood Transfusion
Vox Sanguinis (2017)

ORIGINAL PAPER

DOI: 10.1111/vox.12619

Causes of iron overload in blood donors – a clinical study

A. H. Laursen,[1] O. W. Bjerrum,[1] L. Friis-Hansen,[2,3] T. O. Hansen,[2] J. L. Marott[4] & K. Magnussen[5,6]

[1]*Department of Haematology, Rigshospitalet, Copenhagen, Denmark*
[2]*Center for Genomic Medicine, Rigshospitalet, Copenhagen, Denmark*
[3]*Department of Clinical Biochemistry, Nordsjællands Hospital, Hillerod, Denmark*
[4]*The Copenhagen City Heart Study, Frederiksberg Hospital, Copenhagen, Denmark*
[5]*Blood Centre Lab, Hvidovre Hospital, Hvidovre, Denmark*
[6]*Department of Immunology and Transfusion Medicine, Sorlandet hospital Kristiansand, Kristiansand, Norway*

Vox Sanguinis

Background and Objectives Despite the obligate iron loss from blood donation, some donors present with hyperferritinaemia that can result from a wide range of acute and chronic conditions including hereditary haemochromatosis (HH). The objective of our study was to investigate the causes of hyperferritinaemia in the blood donor population and explore the value of extensive HH mutational analyses.

Materials and Methods Forty-nine consecutive donors (f = 6, m = 43) were included prospectively from the Capital Regional Blood Center. Inclusion criteria were a single ferritin value >1000 μg/l or repeated hyperferritinaemia with at least one value >500 μg/l. All donors were questioned about their medical history and underwent a physical examination, biochemical investigations and next-generation sequencing of HH-related genes, including the *HFE* gene, the haemojuvelin gene (*HFE2/HJV*), the hepcidin gene (*HAMP*), the ferroportin 1 gene (*SLC40A1*) and the transferrin receptor 2 gene (*TFR2*).

Results Forty of 49 donors were mutation positive with a combined 69 mutations, 54 of which were located in the *HFE* gene. There were 11 mutations in the *TFR2* gene, two mutations in the *HFE2* gene and two mutations in the *HAMP* gene. Only four donors had apparent alternative causes of hyperferritinaemia.

Conclusion HH-related mutations were the most frequent cause of hyperferritinaemia in a Danish blood donor population, and it appears that several different HH-genotypes can contribute to hyperferritinaemia. HH screening in blood donors with high ferritin levels could be warranted. HH-related iron overload should not in itself result in donor ineligibility.

Received: 5 May 2017,
revised 31 October 2017,
accepted 4 November 2017

Key words: blood donors, haemochromatosis, *HFE*, hyperferritinaemia, iron overload.

Introduction

Long-term blood donation can cause depletion of the body's iron stores [1]. Blood donors in the capital region of Denmark undergo regular ferritin measurements to prevent donation-related depletion of iron storages. Interestingly, some donors demonstrate hyperferritinaemia despite regular blood donations. Ferritin is an acute-phase protein, and hyperferritinaemia can result from a wide range of conditions, including acute and chronic infection, malignant disease, autoimmune disorders and excessive alcohol consumption. Elevated serum ferritin concentration is also a potential indicator of iron-overload conditions including hereditary haemochromatosis (HH). Hereditary Haemochromatosis is associated with dysregulated intestinal iron absorption and excessive iron deposition in variant tissues (e.g. liver, heart, joints and endocrine glands) leading to organ dysfunction as a result

Correspondence: Adam Høgsbro Laursen, Department of Haematology, Rigshospitalet, Blegdamsvej 9, 8513, Copenhagen, Denmark
E-mail: adam.hoegsbro.laursen.01@regionh.dk

of cell and DNA damage from iron-related generation of reactive oxygen species [2]. Our understanding of the genetic basis of HH was markedly altered with the discovery of the hepatic protein hormone hepcidin, encoded by the *HAMP* gene [3]. Hepcidin regulates iron transport through interaction with the sole mammalian iron transporter, ferroportin 1 (FPN1), encoded by the *SLC40A1* gene. We now recognize hepcidin as the cornerstone in HH pathogenesis. Several different genetic alterations can cause phenotypical HH by affecting either *HAMP* gene expression or hepcidin function [4]. The vast majority of clinical HH cases are caused by loss-of-function mutations in the *HFE* gene [5, 6], namely the C282Y missense mutation that impairs *HFE*-transferrin receptor 1 (TFR1) interaction leading to reduced *HAMP* gene expression and an increase in iron absorption [3]. C282Y homozygosity has a prevalence of about 1/200 persons with northern European descent and is by far the most frequent genotype in HH [5–7]. Other well-known *HFE* gene defects, namely H63D and S65C, can contribute to HH pathogenesis, but clinical HH will typically only develop with the simultaneous presence of the C282Y mutation or other risk factors [3, 8]. Non-*HFE* genotypes can also give rise to phenotypical HH, thus adding to the heterogeneity of HH pathogenesis [9–11]. In addition to *HAMP* and *SLC40A1* mutations, clinical HH can develop from alterations in the *HFE2* and *TFR2* genes, both important modulators of the iron-sensing machinery [10]. In contrast to the *HFE* mutations, the non-*HFE* mutations are not restricted to individuals of northern European ancestry [12].

Although the paradox of iron overload in blood donors is of great scientific interest, the causes of hyperferritinaemia in these subjects are only sparsely touched upon in the literature. It is unclear how we should approach hyperferritinaemia in blood donors, and when hyperferritinaemia should lead to donor deferral. This study investigates the causes of unexplained hyperferritinaemia while exploring the relevance of extensive HH mutational analyses in a population of Danish blood donors.

Materials and methods

The Capital Regional Blood Center of Denmark has a donor population of 55 000-60 000 and an annual donation number of 90 000-100 000. Donors are routinely screened for iron deficiency, in part through regular ferritin measurements [13] that can also serve to detect hyperferritinaemia. In this study, inclusion criteria were either a single ferritin value above 1000 μg/l or repeated hyperferritinaemia (for both genders defined as values >300 μg/l at ≥ 2 consecutive blood donations including at least one value >500 μg/l). Donors were contacted personally and informed about their test results, the study purpose and the voluntary nature of participation. All contacted donors agreed to participate in the study. From 30 January 2012 to 24 March 2014, 49 consecutive donors (f = 6, m = 43) were prospectively included and referred to the Department of Haematology for initial evaluation. There was no predefined time interval from inclusion to initial evaluation. Despite their hyperferritinaemia, donors were still considered to be healthy, and hence, blood donation could be continued in this time period as long as donors signed the standard form stating to be healthy at donation.

None of the donors had previously been diagnosed with HH. To detect potential causes of hyperferritinaemia, all donors underwent a full physical examination, medical history, HH mutation analyses and biochemical investigations including a complete blood count, haemoglobin (Hb), parameters of iron and glucose metabolism, liver and kidney function tests and C-reactive protein (CRP). Further investigations (e.g. abdominal ultrasound, pulmonary function testing, bone marrow biopsy) were performed when relevant in donors with persistent unexplained hyperferritinaemia.

The haemochromatosis gene panel included mutational status of the *HFE* gene and the most common non-HFE genes related to HH [14]: the haemojuvelin gene (*HFE2/HJV*), the hepcidin gene (*HAMP*), the ferroportin 1 gene (*SLC40A1*) and the transferrin receptor 2 gene (*TFR2*). The analysis was carried out by next-generation sequencing using sequence capture to capture all exons from the following transcripts: *NM_021175* (*HAMP*), *NM_000410* (*HFE*), *NM_213653* (*HFE2*), *NM_014585* (*SLC40A1*) and *NM_003227* (*TFR2*). Library construction was carried out from 50 to 500 ng of genomic DNA isolated using the DSP DNA mini kit (Qiagen) according to the instructions by the manufacturer. Adaptor ligation using adaptors from the TruSeq DNA Sample Preparation Kit (Illumina) was performed using the SPRIworks System I for Illumina Genome Analyzer (Beckman Coulter). Sequence capture (Roche NimbleGen) was conducted using the double capture protocol described by the manufacturer. Sequencing was performed on a MiSeq (Illumina) to an average depth of at least 100x. Sequencing data were analysed using Sequence Pilot software (JSI medical systems), and variants were called if the allele frequency was above 25%. All variants were verified by Sanger sequencing on an ABI 3730 DNA Analyzer and numbered according to the GenBank accession numbers using the guidelines of the Human Genome Variation Society (www.hgvs.org/mutnomen).

Donors were divided into four genotype categories: mutation group 1 = classical HH-genotypes (C282Y/C282Y or C282Y/H63D); mutation group 2 = classical HH-genotypes with additional mutations (C282Y/C282Y/x or C282Y/H63D/x); mutation group 3 = others and mutation group 4 = mutation negative.

The integrated ALAMUT VISUAL software for *in silico* data analysis (v.2·8·0) (http://www.interactive-biosoftware.com) including Align GVGD (A-GVGD), PolyPhen-2 and SIFT was used to predict the pathogenicity of specific variants at the protein level. The *in silico* effect of intron variants on splicing was examined as previously described [15]. Default settings were used in all predictions. The frequency of the variants was obtained from the Exome Aggregation Consortium (ExAC) database. Moreover, the frequency was examined in data from 2000 Danish exomes [16]. The variants were classified according to the five-tiered scheme where Class 5 is pathogenic, Class 4 is likely pathogenic, Class 3 is uncertain due to insufficient evidence, Class 2 is likely benign and Class 1 is benign [17].

A subjective risk stratification was performed using ferritin levels, mutation status (C282Y homozygotes at highest risk), sex (females at lowest risk), age (young subjects at lowest risk) and lifestyle factors (e.g. alcohol consumption). After initial evaluation and full mutational analysis, low-risk patients were informed that no follow-up was necessary, but that adherence to the blood donor programme, including regular ferritin measurements, was recommended. Patients with moderate risk were assigned to outpatient follow-up including blood donation at least until normalization of ferritin levels. Patients with high-risk profiles (including persistently high ferritin levels and either high-risk mutational status or unexplained hyperferritinaemia) were treated with therapeutic phlebotomy ± supplementary blood donation. Patients with C282Y homozygosity or C282Y/H63D compound heterozygosity were instructed to inform first-degree relatives about the HH risk. Donors had the option of bringing family members to the follow-up consultation to receive information.

Statistics

Comparison of baseline characteristics between the different mutation categories was done using Fisher's exact test for categorical variables and ANOVA for continuous variables. *P*-values <0·05 were considered significant. Analyses were performed using R version 3·2·0. One subject was lost to observation. Ferritin values for this subject were not included in the statistical analysis.

Results

Study population

At the time of inclusion, all donors were eligible for blood donation. Mean Hb was 15·3 g/dl, and all donors had Hb levels within the reference values. Mean donor age was 42 years of age (range 21–66). The average number of blood donations was 20, covering 33 donors with 10 or fewer donations prior to inclusion and five donors with 50 or more donations.

Average biochemical values not related to iron status were unremarkable for the study population as a whole (data not shown). There were 40 mutation-positive subjects: 13 had classical HH-genotypes (mutation group 1), four had classical HH-genotypes with additional mutations (mutation group 2) and 23 presented with alternative genotypic profiles (mutation group 3). Nine donors were mutation negative (mutation group 4). Table 1 shows genotype-segregated baseline data.

Iron status

The average ferritin level in the study population was 659 μg/l in the blood bank at the time of inclusion and 403 μg/l at the clinical evaluation. Using an upper reference value of 300 μg/l for men and women, 31 of 49 donors still had hyperferritinaemia at the clinical evaluation. Eleven of 49 donors (eight of them from mutation group 1 or 2) presented with elevated transferrin saturation (normal range 0·2–0·5). Nine donors had both hyperferritinaemia and elevated transferrin saturation. Five donors presented with hyperferritinaemia as well as elevations of transferrin saturation and iron levels (four of them from mutation group 1).

Routine laboratory investigations

Three donors had marked alanine aminotransferase (ALAT) elevations, but no other clinical or paraclinical signs of liver dysfunction. Two of these cases were explained by excessive alcohol consumption (≥14 units/week for women and ≥21 units/week for men according to the Danish Health Authority definition) in the immediate period prior to laboratory testing. The third case had a normal abdominal ultrasound, and spontaneous normalization of the ALAT level occurred. One donor (referred to as donor 1) had a moderately elevated alkaline phosphatase level and a marked GGT elevation explained by an excessive alcohol intake. Another donor (donor 2) had a GGT value of five times the upper normal reference, similarly explained by a high weekly alcohol consumption.

Investigations and follow-up

Figure 1 provides an overview of the investigational approach and the therapeutic interventions. Of the 48 donors who showed for clinical evaluation, 17 had normalization of plasma ferritin levels. Two of these donors were without HH-related genetic abnormalities and could

Table 1 Genotype-segregated baseline data of the study population

Variable, unit {reference values}	Mutation group 1 (n = 13)	Mutation group 2 (n = 4)	Mutation group 3 (n = 23)	Mutation group 4 (n = 8)	P-value
Males – % (no./total no.)	76·9 (10/13)	75·0 (3/4)	91·3 (21/23)	100·0 (8/8)	0·333
Age, years – mean (SD) [range]	35·3 (10·7) [21·6–61·2]	43·9 (14·9) [30·7–65·3]	45·1 (11·1) [22·9–66·6]	47·3 (11·5) [30·8–63·6]	0·061
Blood donations – mean (SD) [range]	12 (10) [1–37]	37 (48) [6–109]	17 (24) [4–117]	36 (41) [4–104]	0·149
Ferritin (at inclusion), μg/l {12–300} – mean (SD) [range]	702·6 (261·9) [437·0–1370·0]	671·6 (289·1) [440·0–1070·0]	580·2 (117·0) [406·5–882·5]	806·0 (404·7) [477·0–1650·0]	0·130
Ferritin (initial evaluation), μg/l {12–300} – mean (SD) [range]	504·2 (251·8) [202·0–1040·0]	451·5 (193·6) [313·0–738·0]	322·0 (140·9) [100·0–806·0]	448·1 (215·6) [241·0–853·0]	0·052
Ferritin (difference), μg/l {12–300} – mean (SD) [range]	198·3 (122·0) [45·5–452·5]	220·1 (163·5) [53·0–386·0]	258·2 (142·3) [–156·5–533·5]	357·9 (283·4) [154·0–973·0]	0·222
Time from inclusion to initial evaluation, days – mean (SD) [range]	94·2 (57·0) [48·0–267·0]	149·8 (40·5) [106·0–203·0]	166·9 (102·9) [32·0–432·0]	252·1 (197·5) [56·0–651·0]	0·027
Blood donations from inclusion to initial evaluation – mean [range]	0·4 [0–2]	0·5 [0–1]	0·9 [0–3]	0·9 [0–2]	0·74
Transferrin, μmol/l {24–41} – mean (SD) [range]	25·8 (3·7) [20·7–34·8]	28·2 (4·4) [23·1–33·8]	31·5 (5·5) [18·9–40·5]	32·3 (4·1) [27·6–38·4]	0·005
Transferrin saturation {0·2–0·5} – mean (SD) [range]	0·54 (0·21) [0·33–0·84]	0·52 (0·11) [0·36–0·61]	0·32 (0·15) [0·15–0·84]	0·27 (0·13) [0·16–0·56]	<0·0001
Iron, μmol/l {9–34} – mean (SD) [range]	27·7 (10·0) [15·0–44·0]	28·5 (4·4) [24·0–34·0]	19·5 (9·2) [10·0–51·0]	17·4 (7·5) [10·0–33·0]	0·018
Hb, g/dl {13·4–16·9} – mean (SD) [range]	14·9 (1·0) [13·4–17·2]	14·6 (1·3) [13·1–16·3]	15·0 (1·0) [13·5–16·9]	15·2 (0·6) [14·2–16·3]	0·838

Hb, haemoglobin.
Genotype-segregated baseline data for the study population at the time of clinical evaluation.

be terminated. The remaining 15 patients had HH-related mutations and were either followed in the outpatient clinic while adherent to the blood donor programme (n = 8), terminated due to low risk of iron-overload complications (n = 6) or treated with therapeutic phlebotomy due to blood bank quarantine (n = 1).

Of the 31 donors with persistent hyperferritinaemia, 25 were HH mutation positive. Eight of these donors were treated with repeated phlebotomy, due to marked increases in ferritin levels and high-risk mutation status (five were C282Y homozygotes, one was H63D homozygote, one was C282Y homozygote with an additional *HAMP* mutation and one was C282Y homozygote with additional mutations in both the *TFR2* gene and the *HFE2* gene). Fourteen mutation-positive subjects had low-risk mutations, but high ferritin levels and therefore underwent follow-up including blood donation. The last three mutation-positive subjects were terminated due to marginal elevations in ferritin levels and low-risk mutation status (two C282Y heterozygotes, one H63D heterozygote). Only in two of the mutation-positive donors did we find apparent alternative causes of hyperferritinaemia: a male donor with *TFR2* heterozygosity was diagnosed with rheumatoid arthritis while a female donor with H63D heterozygosity reported excessive alcohol consumption and had ultrasound indices of steatosis (donor 2). Of the six donors with persistent hyperferritinaemia and no HH-related mutations, one had chronic lung disease, one had both chronic lung disease and excessive alcohol intake with ultrasound indices of steatosis and chronic cholecystitis (donor 1), and three subjects were without signs of systemic disease at follow-up and were terminated. The last mutation-negative subject (a 44-year-old male of Korean descent) repeatedly presented with ferritin levels of approximately 1300 μg/l despite regular blood donations. He had normal hepcidin levels and iron deposits in bone marrow biopsy, and abdominal magnetic resonance imaging was unremarkable. Supplementary therapeutic phlebotomies normalized the ferritin level, but the cause of the hyperferritinaemia remains unresolved.

Mutation analysis

The mutation-positive donors combined for 69 mutations, the vast majority (54) of which was found in the *HFE* gene, although we did register 11 mutations in the *TFR2* gene, two mutations in the *HFE2* gene and two mutations in the *HAMP* gene (Table 2). There were 35 donors with 1–2 mutations and only five donors with ≥3 mutations. The study population was quite heterogenic, as we found 18 different genotypes in the mutation-positive subjects. Table 3 provides a detailed list of the specific mutations for each subject.

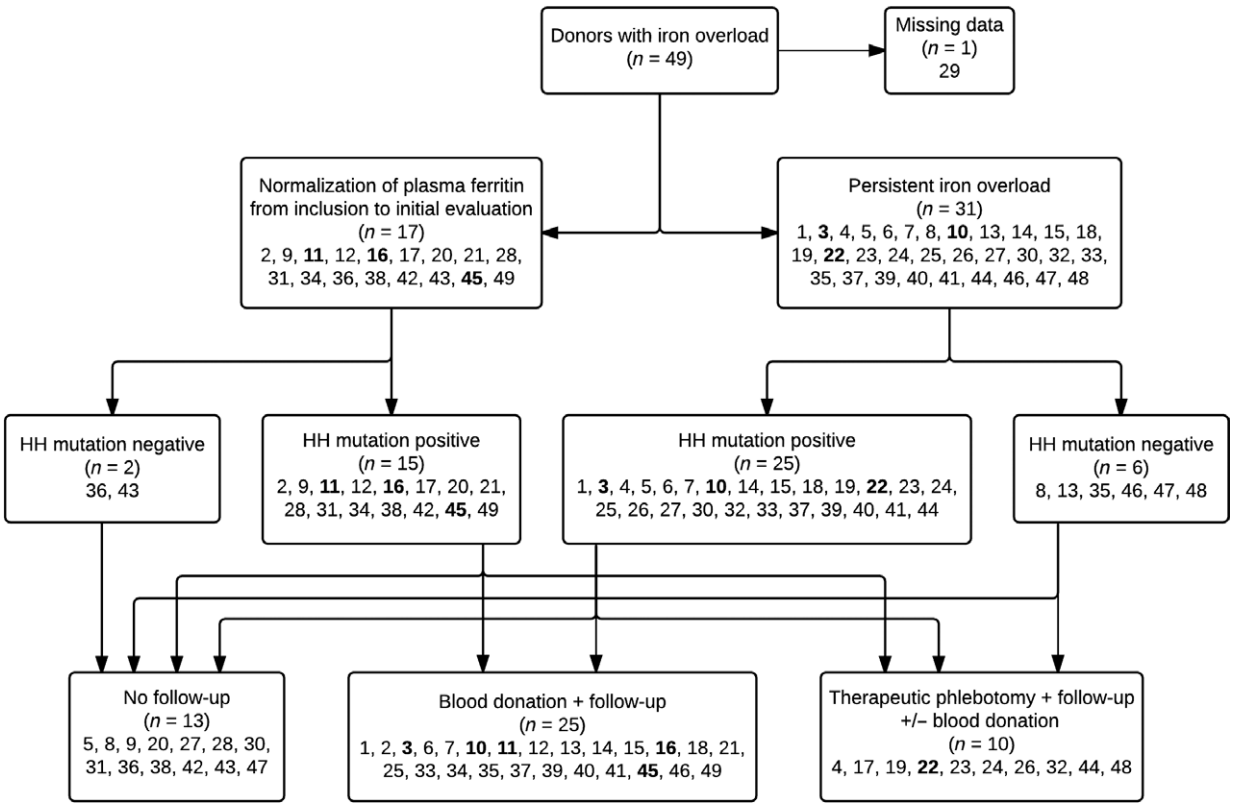

Fig. 1 Flow chart on the investigational approach and follow-up. The numbers represent the donor identification numbers, also used in Table 3 with individual mutation status. Female donors are represented by bold font. HH = hereditary haemochromatosis.

Table 2 *HFE* and non-*HFE* mutations in the study population

HFE			**Non-*HFE***				
C282Y (no.)	**H63D (no.)**	**S65C (no.)**	***HFE2* (no.)**	***HAMP* (no.)**	***TFR2* (no.)**	***SLC40A1* (no.)**	**Total**
33	20	1	2	2	11	0	69

Overview of the HH-related mutations in 49 blood donors.

HFE gene mutations are subdivided into three categories (C282Y, H63D and S65C).

Non-*HFE* mutations include abnormalities of the haemojuvelin gene (*HFE2*), the hepcidin gene (*HAMP*), the transferrin receptor 2 gene (*TFR2*) and the ferroportin 1 gene (*SLC40A1*).

Mutation characteristics

Table 4 (including references [18–26]) provides an overview of the non-*HFE* mutations found in the study population. For each mutation, changes in the nucleotide and amino acid sequence are listed. Mutation type is categorized as missense (a mutation resulting in a codon coding for a different amino acid), synonymous (a mutation resulting in a codon coding for the same amino acid) or intronic (a mutation located in an intron). The predicted effect of the different gene variants on protein function is presented along with classification of pathogenicity. The mutation prevalence in the study population is all higher than reported in comparable population studies, but the low number of donors with mutations do not justify a comparison.

Discussion

The study presents 49 blood donors with asymptomatic hyperferritinaemia. There were only six females, probably due to the obligate blood and iron loss in menstruating women and the inhibitory effect of testosterone on hepcidin synthesis [27]. It should be noted that the same

Table 3 Individual mutation status for all donors in the study population

Donor	Mutations (no.)	Mutations specified
1	2	C282Y/H63D
2	1	*HFE2*(c.9G>C)
3	2	C282Y/C282Y
4	2	C282Y/C282Y
5	1	H63D
6	1	*TFR2*(c.2255G>A)
7	2	C282Y/H63D
8	0	Negative
9	1	H63D
10	1	H63D
11	2	C282Y/C282Y
12	1	*TFR2*(c.1391-6C>T)
13	0	Negative
14	2	C282Y/H63D
15	1	H63D
16	1	H63D
17	2	C282Y/H63D
18	1	C282Y
19	2	C282Y/C282Y
20	1	H63D
21	2	H63D/*TFR2*(c.1473G>A)
22	3	C282Y/C282Y/*HAMP*(c.212G>A)
23	2	H63D/H63D
24	4	C282Y/C282Y/*HFE2*(c.98-6C>G)/*TFR2*(c.2255G>A)
25	3	C282Y/H63D/*TFR2*(c.2255G>A)
26	2	C282Y/C282Y
27	1	C282Y
28	1	C282Y
29	0	Negative
30	2	H63D/*TFR2*(c.458T>C)
31	1	H63D
32	2	C282Y/C282Y
33	3	H63D/*TFR2*(c.1767 + 7C>T)/*TFR2*(c.1770C>T)
34	1	C282Y
35	0	Negative
36	0	Negative
37	2	C282Y/C282Y
38	1	H63D
39	1	*TFR2*(c.1364G>A)
40	3	C282Y/H63D/*TFR2*(c.1767 + 7C>T)
41	1	*TFR2* (c.2255G>A)
42	2	C282Y/S65C (c.193A>T)
43	0	Negative
44	2	C282Y/C282Y
45	2	C282Y/H63D
46	0	Negative
47	0	Negative
48	0	Negative
49	2	H63D/*HAMP* (c.252G>A)

Nucleotide changes for the different *TFR2*, *HFE2* and *HAMP* mutations are shown in parentheses.

C282Y mutations are c.845G>A while H63D mutations are c.187C>G.

Donor identification numbers correspond to the numbers in Fig. 1.

normal range for plasma ferritin was used for both male and female donors. At inclusion, all donors were without known chronic disease, nor signs of organ dysfunction relating to iron overload. Non-HH-related causes of hyperferritinaemia were identified in only four subjects. This indicates that some donors may have been included due to transient hyperferritinaemia. However, only donors with either very high ferritin levels or repeated hyperferritinaemia well over the normal upper reference limit were included. With a minimum interval of 3 months between blood donations (and ferritin measurements), it is unlikely that transient hyperferritinaemia (e.g. acute infection) was a dominant cause of study inclusion. Between inclusion and clinical evaluation, the study population demonstrated a marked reduction in ferritin level with no significant between-group difference. There was a considerable interindividual variation in time from inclusion to clinical evaluation as some donors were seen shortly after their first referral while other donors only showed for clinical evaluation after repeated referrals. Time from inclusion to initial evaluation was highest in mutation-negative subjects, but this finding was most likely a result of chance rather than mutation status and was not accompanied by a significant difference in the number of donations during this period of time (Table 1).

The 40 mutation-positive subjects combined for 18 different genotypes and 69 mutations, the majority of which were expectedly located in the *HFE* gene, including 33 C282Y mutations. However, C282Y homozygosity was present in only 10 (25%) of the mutation-positive donors, which is surprising since clinical iron overload, and risk of iron-overload-related disease is far more common in C282Y homozygotes compared to other HH genetic subpopulations [28, 29]. In a meta-analysis of 32 studies including 2802 HH patients, prevalence of C282Y homozygosity in clinical cases of HH was around 80% [5]. Hence, if all cases of hyperferritinaemia in our mutation-positive donors were indeed caused by the HH-related mutations, we would have expected a larger proportion of C282Y homozygotes. C282Y/H63D compound heterozygosity was found in seven donors (18%), a higher percentage than previously reported in both control populations (1·3%) and in clinically recognized HH (5·3%) [5]. Although two of the individuals (donors 25 and 40) did have additional *TFR2* mutations, the high prevalence of C282Y/H63D compound heterozygotes is surprising, especially considering that none of these donors had apparent comorbidities. The majority of the mutation-positive donors (n = 23) did not present with classical *HFE* HH-genotypes. We found 15 non-*HFE* mutations including two *HAMP* mutations in individuals with C282Y homozygosity and H63D heterozygosity, respectively. Interestingly, one of the *HAMP* mutations – a missense mutation

in exon 3 – has previously been reported as a potential modifier of HH-phenotype in individuals carrying C282Y mutations [18]. There were two *HFE2* mutations, one of them appearing isolated (a missense mutation) while the other (an intronic mutation) was found in an individual with both C282Y homozygosity and a *TFR2* mutation. *HFE2* mutations are mostly associated with juvenile forms of HH, and although there are reports of potentially pathogenic *HFE2* mutations in adult-onset HH, their role in this context is unclear [20, 21, 30]. We found ten donors with a combined total of 11 *TFR2* mutations (seven different variants). Four of these donors did not have any other HH-related abnormalities and even though one of the mutations (c.1364G>A, a missense mutation in exon 10) has been reported as a HH risk factor [22, 23], it is unlikely that the hyperferritinaemic state in these donors could be explained by their genotype alone. The most interesting *TFR2*-mutated donors were donors 21 and 30 (Table 3) who presented with classification 3-mutations in combination with H63D heterozygosity. The c.1473G>A mutation in donor 21 could affect the alternative splicing of RNA [24]. The c.458T>C missense mutation in donor 30 is – to our knowledge – not described previously. Two donors with classical HH-genotypes (donors 24 and 25) presented with presumably benign *TFR2* mutations, and in these cases, the clinical relevance of the *TFR2* mutations is probably negligible. The high number of *TFR2* mutations in our study population was unexpected as prevalence of *TFR2* mutations in clinical HH is low with no more than approximately 30 pathogenic *TFR2* mutations reported worldwide [31]. Most cases of *TFR2*-related HH have been reported in Japan and southern Europe, namely Italy [11, 31, 32]. Interestingly, two of the donors with *TFR2* mutations were indeed of southern European ancestry (donors 30 and 33).

None of the non-*HFE* mutations reported here have documented pathogenicity when appearing isolated, and their contribution to the hyperferritinaemia is therefore uncertain. In general, most of the documented genotypes in the study population would not be expected to cause iron overload. However, for a given genotype, there is a wide range of phenotypic expressions, [33] which indicates that clinical disease expression is dependent on the presence (or absence) of additional genetic and environmental disease modifiers. Similarly, concurrent unrecognized occult disease could contribute to variations in phenotype. Hence, H63D, S65C and non-*HFE* HH-related mutations may contribute to iron overload in the context of other relevant mutations or risk factors. The impact of these mutations could have been assessed through investigation of mutation status and phenotype of the donor relatives.

Our results demonstrate that classical HH-genotypes have a more profound impact on iron metabolism. In our study, eight of 11 donors with elevated transferrin saturation were either C282Y homozygotes or C282Y/H63D compound heterozygotes. In addition, only in three of the 17 donors (18%) with classical HH-genotypes did normalization of ferritin levels from inclusion to initial evaluation occur. In contrast, 12 of 23 donors (52%) with alternative HH-genotypes normalized their ferritin in the same period, possibly illustrating that HH treatment is more effective in this group than in donors with classical *HFE* genotypes.

Despite a high prevalence of HH-related genetic alterations in blood donors with hyperferritinaemia, it is questionable if there is a treatment-related or health-economic rationale for performing routine HH screening in this population. Considering the high prevalence of HH, numerous individuals with the disease are undoubtedly already blood donors and thus undergoing *de facto* HH treatment [34]. However, screening of blood donors with hyperferritinaemia could represent a simple and cost-effective way of identifying only those with a high probability of clinical HH since iron overload in healthy individuals adherent to the blood donor programme could indicate significant malfunction of iron metabolism [35]. Screening could also identify subjects with normal mutation status and hyperferritinaemia who may benefit from a diagnostic workup to reveal serious causes of elevated ferritin levels [35, 36].

However, screening of hyperferritinaemic donors is not uncomplicated since a HH diagnosis could result in permanent donor deferral due to the identification of chronic disease. Furthermore, one could argue that accepting HH donors challenges the altruistic principle of blood donation since these individuals obtain personal benefit from adherence to the donor programme. Still, the perspective of accepting asymptomatic HH donors is intriguing especially given the fact that there is no evidence to suggest that blood from these donors should be of inferior quality compared to non-HH donors [34]. Although some countries continue to have strict rules against transfusion with HH donor blood, several other countries have included uncomplicated HH patients in the donor population [36, 37].

The approach to blood donors with hyperferritinaemia remains a challenge. The present study demonstrates that in most cases, the causes of hyperferritinaemia in blood donors can indeed be found. To our knowledge, this is the first time such a high frequency of HH-related mutations has been reported in blood donors. Although further evidence is required, it appears that HH is not strictly a monogenic disease and that a HH-phenotype can result from the combined effects of several different genetic modifiers [18]. There are obvious benefits to HH screening and inclusion of asymptomatic HH patients, such as earlier diagnosis, less morbidity and improved adherence to the blood donor programme [37]. Inclusion of

Table 4 Overview of the non-*HFE* mutations in the study population

Gene	Nucleotide change	Protein change	Mutation type	Study population prevalence (%)	Population prevalence (%) (NFE/Danes)	Align A-GVGD/SIFT	MaxEntScan	References	Classification
HAMP	c.212G>A	p.Gly71Asp	MS	2·0	0·35/1·03	C0/Tolerated	NA	[18, 19]	3
HAMP	c.252G>A	p.Thr84Thr	Syn	2·0	0·13/0·15	NA	NA	[18]	2
HFE2	c.9G>C	p.Glu3Asp	MS	2·0	NA/NA	C0/Tolerated	NA	This study	3
HFE2	c.98-6C>G	-	Intronic	2·0	NA/NA	NA	SA:11·06/10·25 (−7·3%)	[19–21]	2
TFR2	c.458T>C	p.Leu153Pro	MS	2·0	0·013/NA	C45/Deleterious	NA	This study	3
TFR2	c.1364G>A	p.Arg455Gln	MS	2·0	0·30/0·77	C35/Deleterious	NA	[19, 22, 23]	3
TFR2	c.1391-6C>T	-	Intronic	2·0	0·0061/NA	NA	SA:12·4/10·7 (−13·7%)	This study	2
TFR2	c.1473G>A	p.Glu491Glu	Syn	2·0	0·48/0·51	NA	SD:3·1/NI (−100%)	[24]	3
TFR2	c.1767 + 7C>T	-	Intronic	4·1	0·83/1·13	NA	SD:7·66/7·66 (0%)	[19]	2
TFR2	c.1770C>T	p.Asp590Asp	Syn	2·0	0·85/0·36	NA	SA:9·88/9·84 (−0·4%)	[25]	1
TFR2	c.2255G>A	p.Arg752His	MS	8·2	4·50/3·53	C25/Deleterious	NA	[19, 26]	1

MS, missense variant; NA, not assessed; NFE, non-Finnish Europeans; NI, not identified, Syn, synonymous variant.

A-GVGD and SIFT are software programs that predict effect of missense variants on protein function. The A-GVGD and SIFT scores (C0, C15, C25, C35, C45, C55 and C65/tolerated, deleterious) reflect increased probability for a variant to affect protein function. MaxEntScan is a software program that predicts the effect of nucleotide change on mRNA splicing.

The thresholds represent score predicted for wild-type sequence/score predicted for mutated sequence.

The scores indicate the values for splice donor (SD) or splice acceptor (SA) sites, respectively.

Changes relative to wild-type sequences are indicated in %.

The variants were classified according to the five-tiered scheme, where Class 5 is pathogenic, Class 4 is likely pathogenic, Class 3 is uncertain due to insufficient evidence, Class 2 is likely benign and Class 1 is benign.

asymptomatic and otherwise healthy HH donors should be regarded as an elegant way of combining the continuous work to reduce shortage of blood products with effective HH treatment, in particular in countries where HH prevalence is high.

Acknowledgements

AH Laursen, OW Bjerrum and K Magnussen contributed to research design, data acquisition, analysis and interpretation, and AH Laursen wrote the manuscript. L Friis-Hansen, JL Marott and TO Hansen contributed to data acquisition, analysis and interpretation. All authors revised the manuscript and approved the final version. We gratefully acknowledge Karla and Verner Sørensens Research Fund for the financial support.

Conflict of interest

The authors declare no conflict of interest.

Disclaimers

None.

References

1 O'Meara A, Infanti L, Stebler C, *et al.*: The value of routine ferritin measurement in blood donors. *Transfusion* 2011; **51**:2183–8
2 Powell LW, Seckington RC, Deugnier Y: Haemochromatosis. *Lancet* 2016; **388**:706–16
3 Pietrangelo A: Genetics, Genetic Testing, and Management of Hemochromatosis: 15 Years Since Hepcidin. *Gastroenterology* 2015; **149**:1240–51
4 Sham RL, Raubertas RF, Braggins C, *et al.*: Asymptomatic hemochromatosis subjects: genotypic and phenotypic profiles. *Blood* 2000; **96**:3707–11
5 European Association for the Study of the Liver: EASL clinical practice guidelines for HFE hemochromatosis. *J Hepatol* 2010; **53**:3–22
6 Adams P, Brissot P, Powell LW: EASL International Consensus Conference on Haemochromatosis. *J.Hepatol.* 2000; **33**:485–504
7 Beutler E, Felitti VJ, Koziol JA, *et al.*: Penetrance of 845G–> A (C282Y) HFE hereditary haemochromatosis mutation in the USA. *Lancet* 2002; **359**:211–8
8 Adams PC: Epidemiology and diagnostic testing for hemochromatosis and iron overload. *Int J Lab Hematol* 2015; **37**(Suppl 1):25–30
9 McDonald CJ, Ostini L, Wallace DF, *et al.*: Next-generation sequencing: application of a novel platform to analyze atypical iron disorders. *J Hepatol* 2015; **63**:1288–93
10 Badar S, Busti F, Ferrarini A, *et al.*: Identification of novel mutations in hemochromatosis genes by targeted next generation sequencing in Italian patients with unexplained iron overload. *Am J Hematol* 2016; **91**:420–5
11 Pietrangelo A: Non-HFE hemochromatosis. *Semin Liver Dis* 2005; **25**:450–60
12 Pietrangelo A, Caleffi A, Corradini E: Non-HFE hepatic iron overload. *Semin Liver Dis* 2011; **31**:302–18
13 Rigas AS, Pedersen OB, Magnussen K, *et al.*: Iron deficiency among blood donors: experience from the Danish Blood Donor Study and from the Copenhagen ferritin monitoring scheme. *Transfus Med* 2017. https://doi.org/10.1111/tme.12477. [Epub ahead of print]
14 Salgia RJ, Brown K: Diagnosis and management of hereditary hemochromatosis. *Clin Liver Dis* 2015; **19**:187–98
15 Ahlborn LB, Dandanell M, Steffensen AY, *et al.*: Splicing analysis of 14 BRCA1 missense variants classifies nine variants as pathogenic. *Breast Cancer Res Treat* 2015; **150**:289–98
16 Lohmueller KE, Sparso T, Li Q, *et al.*: Whole-exome sequencing of 2,000 Danish individuals and the role of rare coding variants in type 2 diabetes. *Am J Hum Genet* 2013; **93**:1072–86
17 Richards S, Aziz N, Bale S, *et al.*: Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med* 2015; **17**:405–24
18 Merryweather-Clarke AT, Cadet E, Bomford A, *et al.*: Digenic inheritance of mutations in HAMP and HFE results in different types of haemochromatosis. *Hum Mol Genet* 2003; **12**:2241–7
19 Faria R, Silva B, Silva C, *et al.*: Next-generation sequencing of hereditary hemochromatosis-related genes: novel likely pathogenic variants found in the Portuguese population. *Blood Cells Mol Dis* 2016; **61**:10–5
20 Lee PL, Barton JC, Brandhagen D, *et al.*: Hemojuvelin (HJV) mutations in persons of European, African-American and Asian ancestry with adult onset haemochromatosis. *Br J Haematol* 2004; **127**:224–9
21 Mendes AI, Ferro A, Martins R, *et al.*: Non-classical hereditary hemochromatosis in Portugal: novel mutations identified in iron metabolism-related genes. *Ann Hematol* 2009; **88**:229–34
22 Hofmann WK, Tong XJ, Ajioka RS, *et al.*: Mutation analysis of transferrin-receptor 2 in patients with atypical hemochromatosis. *Blood* 2002; **100**:1099–100
23 Lee PL, Barton JC: Hemochromatosis and severe iron overload associated with compound heterozygosity for TFR2 R455Q and two novel mutations TFR2 R396X and G792R. *Acta Haematol* 2006; **115**:102–5
24 Del-Castillo-Rueda A, Moreno-Carralero MI, Cuadrado-Grande N, *et al.*: Mutations in the HFE, TFR2, and SLC40A1 genes in patients with hemochromatosis. *Gene* 2012; **508**:15–20
25 Lee PL, Halloran C, West C, *et al.*: Mutation analysis of the transferrin receptor-2 gene in patients with iron overload. *Blood Cells Mol Dis* 2001; **27**:285–9

26 Mattman A, Huntsman D, Lockitch G, *et al.*: Transferrin receptor 2 (TfR2) and HFE mutational analysis in non-C282Y iron overload: identification of a novel TfR2 mutation. *Blood* 2002; **100**:1075–7
27 Latour C, Kautz L, Besson-Fournier C, *et al.*: Testosterone perturbs systemic iron balance through activation of epidermal growth factor receptor signaling in the liver and repression of hepcidin. *Hepatology* 2014; **59**:683–94
28 Gurrin LC, Bertalli NA, Dalton GW, *et al.*: HFE C282Y/H63D compound heterozygotes are at low risk of hemochromatosis-related morbidity. *Hepatology* 2009; **50**:94–101
29 Feder JN, Gnirke A, Thomas W, *et al.*: A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. *Nat Genet* 1996; **13**:399–408
30 Koyama C, Hayashi H, Wakusawa S, *et al.*: Three patients with middle-age-onset hemochromatosis caused by novel mutations in the hemojuvelin gene. *J Hepatol* 2005; **43**:740–2
31 Radio FC, Majore S, Binni F, *et al.*: TFR2-related hereditary hemochromatosis as a frequent cause of primary iron overload in patients from Central-Southern Italy. *Blood Cells Mol Dis* 2014; **52**:83–7
32 McDonald CJ, Wallace DF, Crawford DH, *et al.*: Iron storage disease in Asia-Pacific populations: the importance of non-HFE mutations. *J Gastroenterol Hepatol* 2013; **28**:1087–94
33 Beutler E: Discrepancies between genotype and phenotype in hematology: an important frontier. *Blood* 2001; **98**: 2597–602
34 Conry-Cantilena C: Phlebotomy, blood donation, and hereditary hemochromatosis. *Transfus Med Rev* 2001; **15**:136–43
35 Waalen J, Felitti VJ, Gelbart T, *et al.*: Screening for hemochromatosis by measuring ferritin levels: a more effective approach. *Blood* 2008; **111**:3373–6
36 Stefashyna O, Stern M, Infanti L, *et al.*: Pattern of care of blood donors with early-uncomplicated hereditary haemochromatosis in a Swiss blood donation centre. *Vox Sang* 2014; **106**:111–7
37 Marrow B, Clarkson J, Chapman CE, *et al.*: Facilitation of blood donation amongst haemochromatosis patients. *Transfus Med* 2015; **25**:239–42