Iron overload in *HFE* C282Y heterozygotes at first genetic testing: a strategy for identifying rare *HFE* variants

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ABSTRACT

Background
Heterozygotes for the p.Cys282Tyr (C282Y) mutation of the *HFE* gene do not usually express a hemochromatosis phenotype. Apart from the compound heterozygous state for C282Y and the widespread p.His63Asp (H63D) variant allele, other rare *HFE* mutations can be found *in trans* on chromosome 6.

Design and Methods
We performed molecular investigation of the genes implicated in hereditary hemochromatosis in six patients who presented with iron overload but were simple heterozygotes for the *HFE* C282Y mutation at first genetic testing. Functional impairment of new variants was deduced from computational methods including molecular modeling studies.

Results
We identified four rare *HFE* mutant alleles, three of which have not been previously described. One mutation is a 13-nucleotide deletion in exon 6 (c.1022_1034del13, p.His341_Ala345>LeufsX119), which is predicted to lead to an elongated and unstable protein. The second one is a substitution of the last nucleotide of exon 2 (c.340G>A, p.Glu114Lys) which modifies the relative solvent accessibility in a loop interface. The third mutation, p.Arg67Cys, also lies in exon 2 and introduces a destabilization of the secondary structure within a loop of the α1 domain. We also found the previously reported c.548T>C (p.Leu183Pro) missense mutation in exon 3. No other known iron genes were mutated. We present an algorithm at the clinical and genetic levels for identifying patients deserving further investigation.

Conclusions
Our results suggest that additional mutations in *HFE* may have a clinical impact in C282Y carriers. In conjunction with results from previously described cases we conclude that an elevated transferrin saturation level and elevated hepatic iron index should indicate the utility of searching for further *HFE* mutations in C282Y heterozygotes prior to other iron gene studies.

Key words: iron overload, hereditary hemochromatosis, *HFE* variants.

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Introduction

Genetic hemochromatosis is one of the most frequent genetics disorders in the Caucasian population. The clinical picture is that of a multisystemic disease. Progressive accumulation and deposition of iron in parenchymal cells can lead to hepatic cirrhosis, diabetes, cardiomyopathy and other complications. Two mutations were initially described within the hemochromatosis gene (*HFE*), namely the p.Cys282Tyr (C282Y) and the p.His63Asp (H63D) mutations.[1] Homozygosity for the C282Y mutation is the most frequent genotype associated with the common adult form of genetic hemochromatosis. C282Y carriers do not usually develop iron overload. A potential role of acquired factors, such as excess alcohol intake, diabetes and liver diseases has been proposed to explain the occurrence of iron overload in those heterozygotes displaying iron overload, although this is controversial.[2-4] On the other hand, an associated genetic defect can be involved. Among these, the more frequent are the compound heterozygous state for C282Y and the widespread p.His63Asp (H63D) variant allele.[1,5,6] Overall compound C282Y/H63D heterozygosity has been reported to account for 2% to 5% of cases of genetic hemochromatosis with a phenotypic expression in published series.[7-9] More rarely, compound heterozygotes for C282Y and the p.Ser65Cys (S65C) allele have been found to display very mild iron overload.[10]

Depending on the population studied, 1.5% to 16.4% of patients presenting with the hemochromatosis phenotype carry only a unique C282Y allele.[7,11-15]

Hemochromatosis in such patients suggests genetic and/or allelic heterogeneity. Indeed, rare alleles have been reported, the majority inherited *in trans* with the C282Y mutation.[16] These rare mutants are referred to as private mutations because they are found occasionally in a small number of individuals usually belonging to the same kindred. In routine clinical practice, the question is how to reach such a diagnosis when subjects with elevated iron indices are simple heterozygotes for the C282Y mutation at first genetic testing and how to decide whether additional genetic studies are required. Here we describe new *HFE* mutations identified during the investigation of patients with C282Y heterozygosity and iron overload. We propose a diagnostic strategy to detect rare *HFE* variants in the light of the findings of the hereafter and previously described cases.

Design and Methods

We investigated six C282Y carriers from four unrelated French families with high iron parameters, including increased levels of serum ferritin (>300 μg/L in men and >200 μg/L in women), high transferrin saturation (>60%) and a hepatic iron index (>1.9 μmol/g/year)[17] measured by magnetic resonance imaging.[18] Amounts of iron removed greater than 5 g for men and 4 g for women were considered significant for defining hereditary hemochromatosis, following the rules of the EASL consensus conference, 2000.[17] All patients gave informed written consent to genetic investigations and database records of iron overload, according to French regulations. The study was approved by the Ethics committee of CHU de Nîmes. A record of the patients' main clinical features related to iron overload was obtained using a standardized form. All the patients were males except one. Two pairs of brothers M2 - M3 and A1 - A2 (Table 1) were referred separately and subsequently recognized as belonging to the same kindred. The common *HFE* genotype, including the determination of the C282Y, H63D and S65C mutations, was analyzed using polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP).[6] DNA sequencing of the exons, exon-intron boundaries and the 5' untranslated region of the *HFE*, *HAMP*, *HJV/HFE2*, *TFR2* and *SLC40A1* genes was undertaken as previously described.[19,20] When a new *HFE* mutant was identified, it was systematically confirmed on a second sample using a sequencing approach or PCR-RFLP if a restriction site was altered by the mutation. Its absence was verified on a sample of 100 unrelated chromosomes of controls without iron overload.

The mutations were studied at the DNA level using *Alamut*[nd] software (Interactive Biosoftware, Rouen, France). Physical and chemical properties of the elongated protein were evaluated using the predict protein server: *http://www.predictprotein.org/about.html* and the instability of the variant by using a specific test according to Guruprasad *et al.*[21] For other variants, molecular modeling was performed using the AMBER 6.0 package as previously described.[12] The coordinates of *HFE* were taken from the 2.6 Angstrom crystal structure[22] and were used as starting geometry for both wild-type and mutant proteins, while the interactions of the α1-α2 domains with transferrin receptor 1 (TfR1) were obtained from the 1 DE4 PDB structure.[23] Molecular visualization was achieved using the VMD program.[24]

Results

We identified four rare *HFE* mutant alleles in six patients (Table 1), three of which have not been previously reported. The probands' age ranged from 26 to 42 years and serum ferritin levels from 345 to 3250 μg/L. Liver iron content was measured in four out of the six subjects and the hepatic iron index was found to be higher than 2 μmol/g/year in all of them.

Patient M1 was 37 years old at diagnosis and had very high serum ferritin levels (3250 μg/L) with an elevated transferrin saturation (62%). Liver iron was not measured at that time, but he underwent regular phlebotomies over 15 years, with a total of 38 during the first 2 years to normalize ferritin levels. This corresponds to an initial removal of about 7.6 g iron. In addition to *HFE* C282Y, this patient had a 13-nucleotide deletion in exon 6 (c.1022_1034del13, p.His341_Ala345>LeufsX119). Although no functional work has been performed, physical and chemical properties revealed instability. Indeed, the instability index for the variant was computed to be 52.85, compared to 40.00 for the wild-type (values greater than 40.00 are considered to lead to instability of a polypeptide molecule). This classifies the p.His341_Ala345>LeufsX119 variant as unstable.

Two brothers, M2 and M3, aged 40 and 42 years, respectively, at diagnosis, were referred and diagnosed independently. Serum ferritin levels were mildly increased (483 and 397 μg/L, respectively) and transferrin saturation was greater than 60%. The hepatic iron index (as determined by magnetic resonance imaging) was 4.8 μmol/g/year in the elder brother and 5.5 μmol/g/year in the younger one. Both underwent phlebotomies. Both brothers were found to be heterozygous for a substitution of the last nucleotide in exon 2 (c.340G>A, p.Glu114Lys). This mutation is located within a loop (110-114: HSKE) between helix 102-109 and strand 116-125. The loss of a negative charge dramatically increases the pI (pI is 6.36 for

the variant and 6.12 for the wild-type) and shows a modification in the relative solvent accessibility at the loop interface.

Patients A1 and A2, were also brothers, aged 36 and 26 years, respectively, at diagnosis. Despite the fact that dysmetabolic syndrome has been reported to reduce the amount of iron overload in patients with hereditary hemochromatosis,[25] patient A1, who possibly had an associated dysmetabolic syndrome, had higher iron parameters (serum ferritin = 952 μg/L and transferrin saturation = 80%) when compared to his brother (serum ferritin = 345 μg/L and transferrin saturation = 65%) with no associated dysmetabolic syndrome. Patient A1 underwent phlebotomies with a total of 2.5 g iron removed to decrease ferritin levels down to 50 μg/L. The presence of high transferrin saturation in both brothers and the family history were the reason for referral for genetic analysis. Both brothers had a single copy of an *HFE* exon 2 missense mutation, p.Arg67Cys, *in trans* to *HFE* C282Y. The p.Arg67Cys mutation leads to a loss of positive charge (pI is 6.01 for the variant and 6.12 for the wild-type) which introduces a loss of flexibility of the 61-69 loop in the α1 domain of the protein, resulting in destabilization of the secondary structure in this part of the molecule.

Lastly, patient B1, the only female in this series, was 28 years of age at diagnosis with high serum ferritin (645 μg/L) and transferrin saturation (80%) and a hepatic iron index of 7.7 μmol/g/year as measured by magnetic resonance imaging. Therapeutic phlebotomy was undertaken and was well tolerated. This woman was found to be a compound heterozygote for a previously described c.548T>C (p.Leu183Pro) missense mutation in exon 3 of *HFE* and C282Y.[26]

No mutation was found in the *HAMP*, *HJV(HFE2)*, *TFR2* and *SLC40A1* genes in any of the six patients.

A search of the literature (1999 to 2010) retrieved information on 15 previously described families (20 probands) with compound heterozygosity for C282Y and another private *HFE* mutation (Table 2). Among the previously described patients, there were only three females (male:female sex ratio: 5.7), with mean (±SD) ages of 46.2 (±11.6). The mean serum ferritin was 1022.8 (±657.1) μg/L and the mean transferrin saturation was 88.3 (±10.0) %. In the cases in which liver iron content was reported, the hepatic iron index was always greater than 2 μmol/g/year, with a median of 5.0±2.1 μmol/g/year.

Discussion

In the present study we report six new cases of hemochromatosis from four unrelated families who were compound heterozygotes for the *HFE* C282Y mutation and a private *HFE* mutation *in trans*. Three of these genotypes have not been previously described and are due to three novel allelic variants. One (p.His341_Ala345>LeufsX119) is a frameshift mutation resulting from a 13-base pair deletion in exon 6, while the three others (p.Arg67Cys, p.Glu114Lys, p.Leu183Pro) are missense mutations.

The only female patient in this series bore a mutation (p.Leu183Pro) previously described in two unrelated Dutch probands[26] *in trans* with C282Y. Bioinformatics

Table 1. New patients with undescribed *HFE* mutations *in trans* to C282Y.

Laboratory number	M1	M2	M3	A 1	A2	B1
Clinical and biological data						
Gender	male	male	male	male	male	female
Age at diagnosis (years)	37	40	42	36	26	28
Serum ferritin (μg/L)	3250	483	397	952	345	645
Transferrin saturation (%)	62	64	95	80	65	80
Hepatic iron content (μmol/g)	ND	220	200	ND	85	216
Hepatic iron index (μmol/g/year)	ND	5.5	4.8	ND	3.3	7.7
Clinical data	hypertension, diabetes, hepatomegaly	no	no	joint pain, overweight, hypertriglyceridemia, hypercholesterolemia	no	no
Phlebotomies	yes	yes	yes	yes	no	yes
Total iron removed (g)	~7.6	NA	NA	2.5	NA	NA
HFE* mutations						
NM_000410.3 : c.[845G>A] +	c.[1022_1034del13]	c. [340G>A]		c.[199C>T]		c.[548T>C]
NP_000401.1 : p.[Cys282Tyr] +	p.[His341_Ala345>LeufsX119]	p.[Glu114Lys]		p.[Arg67Cys]		p.[Leu183Pro]
Location of the new mutation	exon 6	exon 2		exon 2		exon 3
Type of mutation	frameshift mutation	missense mutation		missense mutation		missense mutation[26]
Consequence (molecular modeling, this study)	13 bp del + 119 aa extension: unstable protein	increases predicted pI and affects relative solvent accessibility within the loop 110-114:HSKE		destabilization of the α1 domain structure		loss of flexibility of the 61-69 loop in the α-1 domain

*Hepatic iron index = hepatic iron concentration/age, NA: not available, ND: not determined *Name of mutations conforms to HGVS nomenclature*

analysis of the p.Leu183Pro mutant showed that this mutation is likely to disturb the interaction between the HFE protein and TfR1.[26] A founder effect has been suggested on the basis of haplotype analysis of this mutation; however, we do not know if the French proband had Dutch ancestors. Our female patient was 28 years old when she was diagnosed with marked iron overload (hepatic iron index: 7.7 μmol/g/year).

It should be noted that 17 out of 20 (85%) compound heterozygotes for C282Y and another *HFE* allelic variant (excluding the C282Y/H63D genotype) reported in the literature are males. This might indicate that clinical expression is milder or absent in females as is the case in C282Y homozygotes.[27-29] Including the cases here, a total of 18 private *HFE* mutations have been described in a compound heterozygous state with C282Y. Analysis of the available reports (Tables 1 and 2) shows that the majority of private mutations are located in exon 2 (8 cases), five mutations are located in exon 3, and three mutations have been found in exon 4. Only one mutation lies in a splice junction (IVS3+1G>T) (Figure 1). We also report here what, to the best of our knowledge, is the first ever described mutant in *HFE* exon 6 (c.1022_1034del13) introducing a frameshift mutation (p.His341_Ala345>LeufsX119). This mutation results in a new protein with a C-terminal extension of 119 amino acids with a stop codon in position 459. The prediction of the p.His341_Ala345>LeufsX119 variant structure could not be built with precision using molecular dynamics technology. An alternative was to use homology modeling programs but none of them is accurate enough to deliver solid results (too many potential structures have been proposed).

The p.Arg67Cys substitution lies in the vicinity of the H63 and S65 residues. The R67 residue is inserted within

Table 2A. Review of the patients with *HFE* mutation *in trans* to C282Y previously described in the literature.

	1	2	3	4	5	6	7
Mutation Initially described as	IVS3+1G>T	G93R	V68fs or V68GfsX20 "t.del203"	E168X	W169X	R71X "R74X"	E168Q/H63D
Reference	39	11	20, 40	41	41	42	32
Clinical and biological data							
Gender	male	male	female	NA	3 males	2 males	male	male
Age at diagnosis (years)	37	40	37	NA	42, 47, 48	50,66	62	40
Serum ferritin (μg/L)	NA	861	NA	NA	608, 1206, 694	1351, 2740	745	540
Transferrin saturation (%)	NA	78	NA	NA	86, 79, 96	100, 106	93	95
Liver iron overload		4+ hepatocyte iron	4+ hepatocyte iron	NA				NA
HIC (μmol/g) HII (μmol/g/year)	HIC: 185 HII: 5.0				HIC: 144, 359, 238 HII: 3.4; 7.6; 5.0	HIC: 421, 425 HII: 8.4; 6.4	HIC: 210 HII: 3.4	
Clinical data	diabetes mellitus, hypogonadism, hepato-splenomegaly	NA	NA	NA	47 y.o. patient: hepatic cirrhosis. 42 y.o. patient: regular blood donor	for both: hepatic cirrhosis, hypogonadism, diabetes. 66 y.o. patient: arthropathy	mild cardiomyopathy, arthritis (hands, elbow, ankles)	no hepatic cardiac or gastro-intestinal dysfunction
Total iron removed (g) (or blood units)	14	(34 units)	NA (blood donor)	3.6, 23, 4	NA, 21	(phlebotomies "started")	NA
HFE* mutations							
NM_000410.3: c.[845G>A] +	c.[616+1G>T]	c.[277G>C]	c.[203delT]	c.[502G>T]	c.[506G>A]	c.[211C>T]	c.[187C>G; 502G>C]
NP_000401.1 p.[Cys282Tyr] +	*not applicable*	p.[Gly93Arg]	p.[Val68GlyfsX20]	p.[Glu168stop]	p.[Trp169stop]	p. [Arg71stop]	p. [His63Asp; Glu168Gln]
Location	IVS 3	exon 2	exon 2	exon 3	exon 3	exon 2	exon 3
Type of mutation	splice	missense	frameshift	nonsense	nonsense	nonsense	double missense
Family screening	one affected identical sibling (sister)	two affected identical siblings	NA	3 probands from the same region of Italy, probable local founder effect	2 probands from the same region of Italy, probable local founder effect	one affected identical sibling (brother, TS 100%, SF: 543 μg/L)	one sibling (sister) and cousins heterozygous for the combined allele and asymptomatic

*SF: serum ferritin, TS: transferrin saturation, HIC: hepatic iron concentration, HII: hepatic iron index (HIC/age), NA: not available, ND: not determined. *Name of mutations conforms to HGVS nomenclature.*

Table 2B. Review of the patients with *HFE* mutation *in trans* to C282Y previously described in literature (continuation).

	8	9	10	11	12	13	14	15
Mutation	L50fs or L50CfsX30	Q283P	W94fs or W94GfsX117	W267fs or W267LfsX80	G43D/H63D	L183P	Y138X	Q233X
Initially described as	"22bp del exon2"		"G93fs"					
Reference	33	12	43	19	31	26	44	35
Clinical and biological data								
Gender	female	male	male	male	male	male	male	female
Age at diagnosis (years)	33	30	62	47	61	44	34	63	34
Serum ferritin (μg/L)	408	395	845	1007	1900	2070	544	1000	474
Transferritin saturation (%)	normal	90	88	69	94	72	83	90	97
Liver iron overload	iron in the liver	ND	iron at liver biopsy (not measured)			NA	NA	NA	"marked hepatocellular iron overload" at liver biopsy
HIC (μmol/g) HII (μmol/g/year)				HIC: 140 HII : 3.0	HIC: 160 HII : 2.6				
Clinical data	overweight, joint pain	NA	NA	NA	NA	pain of small joints	pain of small joints	NA	NA
Total iron removed (g) (or blood units)	(ferritin 11 μg/L after 6 units removed)	3	NA (blood donor, then phlebotomies after diagnosis)	2.75 (11 phlebotomies but blood donor for 10 years)	> 6g (32 units over 2 years)	NA	2.3 (9 units of 400 mL)	NA	(erythrocytapheresis +rHuEPO)
HFE* mutations								
NM_000410.3 c.[845G>A] +	c.[149_170del22]	c.[848A>C]	c.[277delG]	c.[794dupA]	c.[128G>A;187C>G]	c.[548T>C]	c.[414C>G]	c.[697C>T]
NP_000401.1 p.[Cys282Tyr] +	p.[Leu50 CysfsX30]	p.[Gln283 Pro]	p.[Trp94 GlyfsX117]	p.[Trp267 LeufsX80]	p.[Gly43Asp; His63Asp]	p.[Leu183Pro]	p.[Tyr138Stop]	p.[Gln233X]
Location	exon 2	exon 4	exon 2	exon 4	exon 2	exon 3	exon 3	exon 4
Type of mutation	frameshift	missense	frameshift	frameshift	double missense	missense	nonsense	nonsense
Family screening								
	no affected identical sibling Mother 64 y.o. with the same genotype and asymptomatic	one affected identical sibling (sister 37 y, TS: 74%, SF: 99 μg/L)	no affected identical sibling (brother C282Y heterozygote)	no affected identical sibling (3 daughters, aged 21, 24 and 19, carriers of the mutant/wt for C282Y are asymptomatic)	no affected identical sibling (siblings simply heterozygotes with the new variant had elevated TS and normal SF)	NA	NA	one affected identical sibling (bother, TS: 90%, SF: 1600 μg/L

*SF: serum ferritin, TS: transferrin saturation, HIC: hepatic iron concentration, HII: Hepatic iron index (HIC/age), NA: not available, ND: not determined. *Name of mutations conforms to HGVS nomenclature.*

a loop, ESRR (mutated for ESRC) which is well conserved in different species. It has been shown that mutants H63D and S65C bind TfR with affinities similar to that of wild-type HFE.[30] Using molecular modeling we confirmed that Arg67 makes no direct contacts at the HFE-TfR interactions. Nevertheless, as already described for the p.Arg66Cys substitution,[15] the p.Arg67Cys mutation might have functional relevance due to the destabilization of the 61-69 loop.

It is noteworthy that 17 out of 18 private *HFE* mutations described so far in conjunction with C282Y (including those presented here) are highly deleterious: 12 of the 18 (67%) are *null* mutations including nonsense, frameshift or mutants pertaining to consensus splice sites and at least five are missense mutations occurring at critical residues involved in HFE-TfR1 binding[23] at positions 43, 93, 168 and 183[11,26,31,32] of the protein sequence or leading to a complete destabilizing effect on the tertiary structure of the HFE protein (p.Q283P)[12] (Tables 1 and 2). This may explain why the resulting phenotype is as severe as or more severe than the phenotype of C282Y homozygotes among the affected patients. It is also worth noting that the mean age at diagnosis was relatively younger in our group of patients compared to that of the patients reported in the literature (Table 2) (35.2±6.1 years *versus* 46.8±11.5 years, respectively). While this may be a consequence of the more severe genotype it may also reflect a greater awareness of the disorder, resulting in more accurate and earlier detection in recent years. Most of the rare *HFE* mutations were identified following two main different approaches: (i) by chance when the new mutation modified the pattern of detection of one of the two common *HFE* mutations (for example, modification of a restriction site[31,33] or denaturing high performance liquid chromatographic pat-

terns[12] and (ii) by further investigation in patients with genetic hemochromatosis carrying at least one chromosome without a common assigned *HFE* mutation.[12] The latter group includes the simple C282Y heterozygotes, with a discrepant phenotype of iron overload.

The vast majority of C282Y carriers will not develop iron overload and can be reassured.[1,2] A careful step by step strategy at the clinical and genetic levels may allow those heterozygotes deserving further investigation to be targeted. The first step consists of clinical examination and assessment of iron parameters (serum ferritin and transferrin saturation) to identify C282Y heterozygotes with an abnormal iron status (Table 3). Once extrinsic factors such as heavy alcohol intake or viral infections have been ruled out, the second step is to search for the H63D mutation, since the presence of this mutation may help to explain an elevation of the iron indices. Because serum ferritin may be increased due to a variety of causes unrelated to iron overload, the third step is to assess hepatic iron stores directly. Magnetic resonance imaging is then necessary to authenticate high hepatic iron content. Liver biopsy is indicated if it can supply information that imaging or blood tests cannot and that will help with the patient's management. Another use is in clinical research and in circumstances in which reliable quantitative magnetic resonance imaging is not available. Benefits and risks for the individual patients should be weighed. In a fourth step, *HFE* sequencing can be undertaken and may identify new

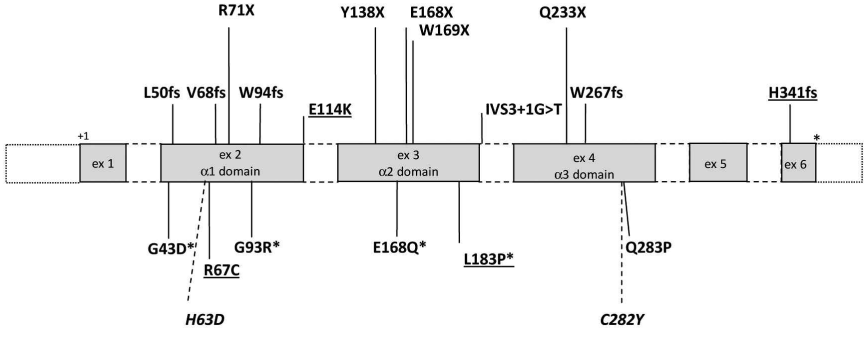

Figure 1. Known and new private mutations described on the *HFE* gene *in trans* to C282Y. Schematic representation of the *HFE* gene showing the location of the mutations described in association with p.C282Y (*in trans*). Nonsense, frameshift and splice mutations are in the upper panel, whereas missense mutations are in the lower panel. Newly identified mutations (this study) are underlined. Most identified missense mutations (indicated by an asterisk) affect key residues presumably involved in HFE-TfR1 binding thus leading to severely disturbed protein function. The p.Q283P mutation is predicted to destabilize the HFE protein.[12] α1, α2 and α3 represent the three extracellular domains of the HFE protein.

Table 3. Step strategy for the diagnosis of rare *HFE* mutations associated with C282Y in a compound heterozygous state.

Step	Action undertaken
Step 1	**Basic biology and clinical examination**
	• Hyperferritinemia in an adult (male > 300 μg/L, female > 200 μg/L)
	• High transferrin saturation (male > 60%, female > 50%)
	• Exclude acquired causes: hematologic disorder, alcohol, cell necrosis.
Step 2	**Basic genetic testing**
	• Test *HFE* (C282Y)
	• C282Y homozygote: *HFE* hemochromatosis: stop (unless very severe iron overload, follow step 4-2)
	• C282Y heterozygote
	o Test *HFE* (H63D)
	- C282Y/H63D compound heterozygote + mild iron overload: stop
	- C282Y/H63D compound heterozygote + high iron parameters: follow step 3
	- No H63D: follow step 3
	• Other *HFE* genotypes: follow step 3
Step 3	**Quantification of hepatic iron (magnetic resonance imaging or liver biopsy*)**
	1. HII≤ 1.9 μmol/g/year:[17] stop
	2. HII> 1.9 μmol/g/year: follow step 4
Step 4	**Specialized genetic testing**
	• Sequence the *HFE* gene:
	1. Presence of a private *HFE* mutation (can explain iron overload)
	2. No additional *HFE* mutation: sequence other iron-related genes (*HAMP*, *HFE2/HJV*, *TFR2*, *SLC40A1*)

*HII: hepatic iron index (hepatic iron content/age); *Magnetic resonance imaging is the preferred non-invasive method where validated quantitative imaging is available. However, it is not able to define cellular distribution of iron within the hepatic lobule. Liver biopsy can be performed in specific cases following a specialized indication, mainly for the diagnosis and prognosis of liver damage.[36]*

HFE variants, as described here.

It should be noted that a high transferrin saturation is a particularly valuable indicator for the presence of a *HFE* mutation and that patients with uncommon compound genotypes have significantly higher levels of transferrin saturation than the usual cut-off for genetic hemochromatosis (i.e. usually > 45%).[34,35] Indeed, we calculated a mean transferrin saturation of 88.3±10.0% from 20 published cases (shown in Table 2 A and B), and 84.5±12.3% when including the six patients described in this study. These results confirm that the findings of high transferrin saturation in such patients together with elevated amounts of hepatic iron are important indicators of the utility of first searching for further *HFE* mutations in C282Y heterozygotes prior to conducting other iron-related gene investigation. This also explains our choice of a high transferrin saturation threshold (60% in adult males and 50% in adult females) as an inclusion criterion (see *Design and Methods* and Table 3) rather than the usual threshold of greater than 45%. These thresholds have already been used in similar circumstances for the detection of *HFE* compound heterozygotes.[12] In our experience using lower transferrin saturation thresholds leads to many unnecessary genetic analyses. It is worth noting that recent studies on screening for *HFE* C282Y homozygotes also used transferrin saturation cut-offs higher than 45%: greater than 50% in men[36] or greater than 50% in women and greater than 55% in men.[37]

In a recently published series it was calculated that 33% of p.Cys282Tyr heterozygous patients with significant iron overload had a rare mutation in *HFE*.[35] Detecting these new mutants has both biological and clinical implications: insight on the functional domains of the HFE protein and management of iron overload in the probands including family screening and genetic counseling among relatives.

Lastly, patients who have no additional *HFE* mutant alleles, will deserve further investigation of other genes implicated in iron overload in order to search for digenism or multigenism.[38] This will include analysis of *HAMP*, *HJV*, *TFR2* and *SLC40A1*. However in clinical practice, once hepatic iron overload has been proven, phlebotomy must be initiated rapidly without waiting for sequencing results. Quantification of the total iron removed by phlebotomies may serve as an additional argument for retrospective evaluation of the extent of iron accumulation.

Authorship and Disclosures

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