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Hereditary Hypotransferrinemia can lead to elevated transferrin saturation and, WHEN associated to *HFE* or *HAMP* mutations, to iron overload

Running Title: Hypotransferrinemia

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Conflict of interest:

The authors state that there is no conflict of interest relevant to this manuscript.

Author's contribution:

BEMP and DJB gathered and analyzed the data and critically reviewed the manuscript. RM performed the biochemical analysis. YD and PB recruited the patient, analyzed the data and reviewed the manuscript. JAM and BEMP performed the genetic testing. LO analyzed the data and reviewed the manuscript. BJE gathered and analyzed the data and wrote the manuscript.

Abbreviations:

NTBI: non-transferrin bound iron

LPI: labile plasma iron

LIC: liver iron content

MRI: magnetic resonance imaging

SNP: single nucleotide polymorphism

TFsat: serum transferrin saturation

ACCEPTED MANUSCRIPT

Abstract

As our understanding of iron metabolism improves through the more accurate description of iron metabolism actors, new causes of iron overload are identified. We, here, report 16 cases of hereditary hypotransferrinemia related to 4 previously undescribed *TF* (transferrin) mutations (p.Val221Gly, p.Arg609Trp, p.Glu370Lys, p.Tyr533X and p.Cys421Arg). We show that, besides increasing serum transferrin saturation without iron overload, hypotransferrinemia, when associated to mutations in *HFE* or *HAMP* or to acquired factors, can lead to clinically relevant iron burden. These cases emphasize the usefulness of serum transferrin determination in the diagnostic evaluation of iron overload and the importance for clinicians to be aware of this syndrome.

Introduction

Understanding of iron metabolism has made important progress as new genes and their roles have been described in iron metabolism [1]. Mutations of those genes are searched for as part of the diagnosis of primary iron overload [2].

The *TF* gene encodes transferrin which is the iron carrier protein in bloodstream [3]. In the clinical setting, serum transferrin saturation (TFsat) is a pivotal parameter as it represents the biologically available iron, and elevated transferrin saturation is highly suggestive of iron overload.

Atransferrinemia is characterized by undetectable serum transferrin, liver iron overload, severe anaemia, and juvenile onset [4-8]. In accordance with its autosomal recessive transmission, heterozygous relatives showed halved transferrin level but normal haematological parameters. However, data regarding their iron metabolism parameters remains poorly documented.

Genome-wide association studies showed that *TF* variants partly explained variations in serum transferrin levels of asymptomatic subjects, and may affect TFsat and serum ferritin [9]. Moreover, *in vivo* study showed that serum transferrin may have a direct role in hepcidin expression [10]. This suggests that serum transferrin level related to *TF* mutations could impact serum transferrin saturation levels and iron metabolism.

Here, we report, in seven families, 16 cases of hypotransferrinemia related to 4 previously undescribed *TF* mutations. We show that, beside increasing TFsat without iron overload, hypotransferrinemia in association to mutations in *HFE* or *HAMP* can lead to clinically relevant iron overload.

Materials & Methods

TF mutations were screened following the identification of hypotransferrinemia (serum transferrin <1.5g/L) in 7 patients referred for suspected iron overload.

In all patients, conditions able to decrease transferrin concentration such as malnutrition, protein malabsorption, hepatocellular failure, and urinary protein losses were ruled out.

The coding region and intronic flanking sequences of the *TF* gene (RefSeq NM_001063.3) were sequenced using the BigDye terminator cycle sequencing kit on a 3130 Genetic Analyser (Applied Biosystems).

The coding sequences of *HFE*, *HJV*, *HAMP*, *TFR2*, *SLC40A1*, *CP*, *FTL*, *FH1*, were analysed by Next Generation Sequencing (NGS) with the Ion Torrent Ampliseq technology on a Personal Genome Machine.

The potential consequences of *TF* mutations were assessed using the algorithm SIFT (<http://sift.jcvi.org/>) and Polyphen-2 (<http://genetics.bwh.harvard.edu/pph2/bgi.shtml>).

Serum hepcidin levels were determined using enzyme immunoassay (Peninsula Laboratories, Bachem, San Carlos USA). Serum hepcidin/ferritin ratios were calculated as follows: (serum hepcidin/serum ferritin) X100. Non-transferrin bound iron (NTBI) and Labile Plasma Iron (LPI) were determined as previously described [11].

Family screening was advised for probands carrying mutation(s). Written informed consent was obtained from each patient and the study was performed in accordance with the Declaration of Helsinki and with the French regulation on medical genetic diagnosis.

Results

Table I describes the clinical characteristics of the patients.

Patient 1 (proband of family 1, Figure 1) had the p.Val221Gly (c.662T>G) *TF* mutation and the p.Gly71Asp (c.212G>A) *HAMP* mutation. He had increased TFSat and iron overload.

Interestingly, one brother (patient 4) was diagnosed with unexplained iron overload 10 years earlier (serum ferritin was 2500 $\mu\text{g/L}$ ($N < 300\mu\text{g/L}$) at diagnosis; he had weekly phlebotomies for several months and maintenance phlebotomy since then). Patient 4 harboured both mutations (p.Val221Gly in *TF*, p.Gly71Asp in *HAMP*). Moreover, he was a compound heterozygote for the p.Cys282Tyr and p.His63Asp mutations in *HFE*. Family 1 pedigree suggests a good segregation between mutation in both *TF* and *HAMP* and the development of iron overload. This phenotype could even be more severe when associated with *HFE* mutation. Relatives with *TF* mutation and *HFE* compound heterozygosity only, did not develop iron overload (Patients 3 and 5), neither did relatives with the sole *HAMP* mutation (Figure 1: patients III.1 and III.3).

Patient 6, and patients 7, 8, 9 belonged to two unrelated families but had the same p.Tyr533X (c.1599_1600del) mutation. Patient 6 had increased TfSat and iron overload. He underwent venesections and 6.2g of iron were removed. Despite having the same serum transferrin levels as patient 6, patients 7, 8 and 9 showed no signs of iron overload. Of note, patient 6 had also the previously described SNP rs1799852 (c.739C>T, p.Leu247Leu)[9].

Patients 10 and 11 had the undescribed p.Cys421Arg (c.1261T>C) mutation. Both had slight iron overload.

Patient 12 had the undescribed splicing mutation c.1623-1 G>T which could induce a loss of exon 14, and the polymorphism p.Leu247Leu. This mutation was absent in his sister who had normal serum transferrin and normal iron parameters.

Patient 13 had the p.Tyr445Cys (c.1334A>G) and the previously described p.Arg609Trp (c.1825C>T) mutation [8]. He had significant iron overload. However, he also had overt alcoholic liver disease and metabolic syndrome. His sister presented the p.Tyr445Cys polymorphism only and had normal serum transferrin and iron parameters. His daughter

(patient 14) had the p.Arg609Trp mutation only and presented slightly decreased serum transferrin.

Patients 15 and 16 had the p.Glu370Lys (c.1108G>A) mutation. Patient 15 was heterozygous for the p.Cys282Tyr *HFE* mutation and presented slight iron overload, while patient 16 had increased transferrin saturation but barely increased serum ferritin.

According to Polyphen-2, *TF* mutations p.Cys421Arg, p.Tyr445Cys, p.Arg609Trp, p.Glu370Lys and p.Val221Gly were probably damaging. According to SIFT, *TF* mutations p.Tyr445Cys, p.Arg609Trp, and p.Val221Gly were deleterious, while *TF* mutations p.Cys421Arg and p.Glu370Lys were tolerated. The *HAMP* p.Gly71Asp mutation was predicted tolerated or benign according to SIFT and Polypehn-2, respectively.

No additional mutations were found in the other sequenced genes. Of note, only patient 2 presented with anaemia which was likely related to her haematological malignancy.

Discussion

To our knowledge, this is the first report describing the consequences of genetic *hypotransferrinemia* on iron metabolism. We show that heterozygous mutations in *TF* can lead to low serum transferrin levels and increased transferrin saturation without affecting erythropoiesis. Moreover, we show that low transferrin levels when associated to genetic or acquired modifiers can lead to iron overload. Overall, our data emphasize the usefulness of serum transferrin level assessment in the diagnosis evaluation of iron overload.

Interestingly, levels of serum transferrin may be different between patients despite carrying identical mutations, suggesting additional, yet unidentified, regulatory factors. However, despite the low levels of functional protein, haematopoiesis was normal confirming haplosufficiency of the *TF* gene.

Contrary to haematopoiesis, our results shows that iron metabolism regulation was impaired in some patients, but serum transferrin was not predictive of iron overload since some patients had both normal serum ferritin values and TFsat. It is noteworthy that TFsat was not strictly correlated to the protein level, suggesting an adaptive mechanism which accordingly would lower serum iron levels.

NTBI and LPI, which could have been raised in this situation of high TFsat, were normal. This may be partly related to the relatively moderate increase of TFsat in most cases (only 3 had Tfsat over 75%) but suggests also that in case of low serum transferrin levels, an adaptive mechanism regulates iron release into the bloodstream to maintain a close to normal transferrin saturation. However, in the presence of genetic or acquired factors, this mechanism could be overwhelmed. The normal serum hepcidin and serum hepcidin/ferritin ratio suggest that this mechanism is not related to hepcidin secretion.

Most of the patients were free of iron overload. Whenever significant iron overload was found it was not clearly correlated with serum transferrin levels or transferrin saturation levels. This may be related to the absence of NTBI and suggests that hypotransferrinemia is only a factor of susceptibility to iron overload, which would be only expressed in the presence of acquired or genetic factors.

Family 1 was especially informative regarding the impact of associated genetic factor. The pedigree clearly showed that simultaneous mutations in *TF* and *HAMP* lead to iron overload in patients without acquired factors. The p.Gly71Asp mutation in *HAMP* has already been described although its relevance remains controversial [12, 13]. However, in our subject, we excluded mutations in other genes involved in iron metabolism, and no acquired factors were associated, which strongly suggests the pathological role of the p.Gly71Asp mutation. The impact of *HFE* compound heterozygosity is more difficult to assess. Its association with the

sole *TF* mutation did not result in iron overload while, when associated with both *TF* and *HAMP* mutations, a more severe disease may develop.

Otherwise, gender may have been another factor leading to the expression of iron overload in our subjects. It is noteworthy that all men had high serum ferritin levels, which is in line with the well-known overexpression of HFE haemochromatosis in males and with the recent finding of hepcidin regulation by testosterone [14].

In conclusion, we suggest that heterozygous *TF* mutation leads to haplosufficiency regarding haematopoiesis but induces susceptibility to iron overload due to haploinsufficiency regarding a still unidentified impact on iron metabolism regulation. On a practical point of view, considering not only transferrin saturation but also serum transferrin level must be the rule when facing with putative iron overload.

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Legends

Figure 1: Pedigree of family 1

Tf: serum transferrin (N:2-3,8 g/L). Frt: serum ferritin ($\mu\text{g/L}$, N<200 in women and <300 in men). Sat: serum transferrin saturation (N<45%).

Arrow indicates the index case.

Table 1 Clinical and biological characteristics of patients

F: family number, P: proband (index case), N: patient number

TF: serum transferrin (g/l, N: 2-3,8), Iron : serum iron ($\mu\text{mol/l}$, N:12,5-25), TFsat: serum transferrin saturation (%), N <45), Ferritin: serum ferritin ($\mu\text{g/L}$, N<200 in women and <300 in men), Hepcidin: serum hepcidin level (nmol/l, N:4-30), hepcidin ferritin ratio (N:4-30), LIC: Liver Iron Content determined by magnetic resonance imaging ($\mu\text{mol/g}$, N<40). NTBI: Non transferrin bound iron (N<0,5 $\mu\text{mol/L}$). LPI: Labile Plasma Iron (N<0,5 $\mu\text{mol/L}$).

* denote value after phlebotomies, initial values are in parentheses.

F	P	N	Sex	Age	Comorbidity	TF	Iron	TF Sat	Ferritin	Hepcidin	Hepcidin /ferritin Ratio	NTBI / LPI	LIC	Transferrin	HFE	HAMP
1	P	1	M	67	BMI 25	1.1	14.7	53	611	22.2	4	<0,5	170	p.[Val221Gly];[=]	p.[=];[=]	p.[Gly71Asp];[=]
		2	F	76	High grade Non Hodgkin Lymphoma	0.95	10.4	43	1527	22.5	2.1			p.[Val221Gly];[=]	p.[Cys282Tyr];[=]	p.[=];[=]
		3	F	64		1	16	64	325	16.4	5	<0,5		p.[Val221Gly];[=]	p.[His63Asp];[Cys282Tyr]	p.[=];[=]
		4	M	73	bloodletting since 2004	1.2	15.5	52* (95)	96* (2500)	2.1	2.2			p.[Val221Gly];[=]	p.[His63Asp];[Cys282Tyr]	p.[Gly71Asp];[=]
		5	M	74		1.21	17.5	58	90					p.[Val221Gly];[=]	p.[His63Asp];[Cys282Tyr]	p.[=];[=]
2	P	6	M	52		1.3	17.9	55	886				170	p.[Tyr533X];[=]	p.[=];[=]	p.[=];[=]
3	P	7	F	70		1.07	20.4	76	143					p.[Tyr533X];[=]	p.[His63Asp];[=]	p.[=];[=]
		8	F	47		1.3	25	75	154					p.[Tyr533X];[=]	p.[His63Asp];[Cys282Tyr]	p.[=];[=]
		9	F	61		1.5	20.6	54.9	44					p.[Tyr533X];[=]	p.[His63Asp];[=]	p.[=];[=]
4	P	10	M	39	BMI 26	1.3	22.4	68	665	26.6	4.3	<0,5	75	p.[Cys421Arg];[=]	p.[=];[=]	p.[=];[=]
		11	M	51		1.25	9.8	31.7	934	28.3	3.1	<0,5	70	p.[Cys421Arg];[=]	p.[=];[=]	p.[=];[=]
5	P	12	F	70	HTA, Dyslipidemia, BMI 32, regular blood donation	1.3	10.6	32	542	39	7.7	<0,5		c.1623-1G>T (splicing mutation)	p.[=];[=]	p.[=];[=]

6	P	13	M	63	Dyslipidemia, diabetes, alcoholic liver disease	0.9	12.4	55	2088			270	p.[Arg609Trp]; [p.Tyr445Cys]	p.[=];[=]	p.[=];[=]
		14	F	28		1.7	16.6	39.7	258				p.[Arg609Trp];[=]	p.[Cys282Tyr];[=]	p.[=];[=]
7	P	15	M	51		1.2	17.6	58	700			90	p.[Glu370Lys];[=]	p.[Cys282Tyr];[=]	p.[=];[=]
		16	F	49	Dyslipidemia	1.12	22	79	288				p.[Glu370Lys];[=]	p.[=];[=]	p.[=];[=]

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F: family number, P: proband (index case), N: patient number

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* denote value after phlebotomies, initial values are in parentheses.

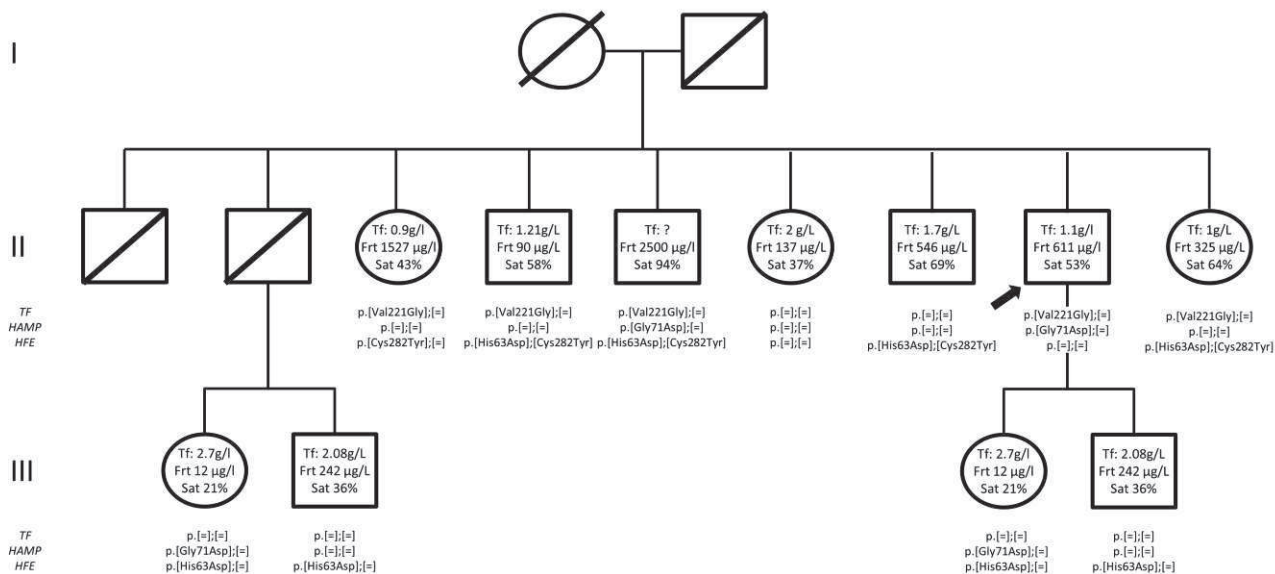


Figure 1: Pedigree of family 1

TF: serum transferrin (N:2-3,8 g/L). Frt: serum ferritin (μg/L, N<200 in women and <300 in men). Sat: serum transferrin saturation (N<45%).
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