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A case series of patients with β-thalassemia trait and iron overload: from multifactorial hepcidin suppression to treatment with mini-phlebotomies

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Short running title: Iron overload in β-thalassemia trait

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CASE REPORTS & CASE SERIES

Beta-thalassemia trait (βTT), which results from heterozygous inheritance of a mutation in the β-globin gene, is characterized by microcytosis and hypochromia with borderline anemia caused by reduced hemoglobin A (HbA) synthesis.[1] Although βTT is typically asymptomatic and benign, subjects display mild ineffective erythropoiesis (IE) and may develop multifactorial suppression of hepcidin, the key regulator of iron homeostasis[2], sometimes leading to clinically relevant iron overload (IO). Prompt diagnosis and management are essential to avoid severe organ damage. However, treatment of IO in βTT can be challenging, as the standard phlebotomy regimen (400-500 ml weekly) used in hemochromatosis (HC) is not feasible in mildly anemic individuals. Furthermore, no specific guidelines about oral iron chelators (deferasirox or deferiprone) exist, and their off-label use may be contraindicated in patients with medical comorbidities (such as chronic kidney disease). Desferioxamine may be an option, but it is cumbersome for the patient and difficult to organize, requiring subcutaneous pump infusion.

Here, we describe a series of 30 βTT patients referred to our Center for Iron Disorders for hyperferritinemia, who were ultimately diagnosed with IO. All participants provided informed written consent, and the study was approved by the local ethics committee. The relevant hematological, biochemical, genetic, and clinical data of patients, as well as the liver iron concentration (LIC) detected by magnetic resonance imaging (MRI) or liver biopsy, are summarized in Table 1. All subjects were Caucasians (predominantly males, 73%), with a median age at diagnosis of 61±10 years. Mild microcytic anemia was present in 90% of subjects (mean Hb 115±11 g/L). Ferritin levels were highly variable, ranging from 450 to over 3,500 µg/L, with 46% of subjects exceeding the threshold strongly associated with iron-related organ damage such as advanced liver fibrosis (> 1,000 µg/L). IO was suggested by elevated transferrin saturation (TSat; >45%) and confirmed, in most subjects, by increased LIC at MRI (>60 µmol/l according to the Gandon's protocol or < 6 ms according to T2* approach), or liver biopsy (Perls' staining showing iron deposits in hepatocytes from 2+ to 4+). After excluding patients with HFE-related HC (homozygous for the C282Y variant), we observed a mild hepcidin increase (evaluated by a mass spectrometry-based method) in the majority of βTT subjects compared to age- and sex-matched healthy individuals from the general population.[3] However, the hepcidin-to-ferritin ratio, which better reflects the appropriateness of hepcidin response to the degree of iron loading, was markedly reduced in our cohort (median 8.9 pmol/µg; Q1-Q3 2.1-13.7 pmol/µg), in comparison with the historical controls[3], suggesting an insufficient hepcidin production in these βTT patients.

Hepcidin inhibits iron absorption and recycling by blocking ferroportin and is physiologically regulated by iron stores through a classical feedback loop.[2] In addition to iron, numerous cofactors can influence hepcidin production in individual patients, with either positive or negative effects.[4] Stimulators include iron replacement therapy, inflammation, and systemic infections, while suppressors include hypoxia, genetic variants, erythropoietic drive, alcohol, advanced liver diseases (especially due to hepatitis C virus), and administration of sex hormones testosterone and estrogens.[4]

Except for HC patients (where primary hepcidin suppression has been expected), the cause of relative hepcidin deficiency and IO was multifactorial in most of our βTT cohort. In particular, we observed significant alcohol consumption (i.e., > 4 alcohol units per day) in 43% of cases.

The H63D variant in the HFE gene was detected in various combinations (homozygous, heterozygous, or in compound heterozygosity with the C282Y) in one-third (33%) of the patients. The role of such a variant is controversial. Some studies have reported higher ferritin levels in βTT subjects that were H63D homozygous or even heterozygous compared to non-carriers.[5,6] This may indicate that co-inheritance of the H63D variant does represent a risk factor for IO in βTT. On the other hand, a study performed on North Indians did not confirm such a synergic effect, but it should be noted that the prevalence of the H63D variant is low in that population.[7]

A next-generation sequencing (NGS) analysis of a panel of 65 iron genes, including hemojuvelin (HJV), hepcidin (HAMP), transferrin receptor 2 (TfR2), and ferroportin (SLC40A1), revealed no pathologic variants in all but one patient, who carried a potentially pathogenic variant (p.*Glu112Gln* or *E112Q*) in the exon 1/pro-peptide domain of the *BMP6* gene.[8] A patient affected by HFE-related HC also presented two potentially pathogenic mutations on the ceruloplasmin (*CP*) gene and reduced CP levels.

Liver cirrhosis was detected in 4 subjects and hepatitis C in 2. Metabolic dysfunctions were detected in a large proportion of our cohort: over half of these βTT patients had at least one dysmetabolic feature (including hypertension, dyslipidemia, impaired fasting glucose or diabetes, hyperuricemia, or increased body mass index), and 27% had two or more features. Furthermore, fatty liver disease, with or without associated inflammatory infiltrate and fibrosis, was detected in 8 patients at liver biopsy.

Dysmetabolic hyperferritinemia represents the most common cause of referral for high ferritin levels in clinical practice.[9] Some of these patients have mildly increased iron stores, a condition also named "dysmetabolic iron overload syndrome" (DIOS), especially in the presence of other risk factors, such as genetic variants, male sex, moderate alcohol intake, and βTT. The underlying pathophysiology is likely multi-factorial due to both environmental and genetic factors. From a mechanistic point of view, lipotoxicity is thought to cause an inefficient block of intestinal iron absorption by hepcidin, and iron retention in hepatocytes also because of reduced ferroxidase activity of ceruloplasmin, overall resulting in liver iron accumulation.[9]

Noteworthy, two patients had been treated with IV iron replacement therapy during the fertile period because of microcytic anemia that was misdiagnosed as due to iron deficiency.

Finally, to better explore the role of ineffective erythropoiesis in our cohort, we measured the levels of erythroferrone (ERFE) in 18 out of the 30 βTT patients by an immunoassay (ELISA).[10] ERFE is a soluble mediator produced by an expanding pool of erythroblasts in the bone marrow, which suppresses liver hepcidin production.[11] Increased ERFE has been associated with the development of IO in anemic patients with IE without erythrocyte transfusions, such as in subjects with beta-thalassemia intermedia (reviewed in Guerra et al[12]).

In our βTT subjects, the median ERFE was 37.9 ng/mL (Q1-Q3 15.3-47.5), a value higher than those previously reported in healthy blood donors but lower than those detected in non-transfused β-thalassemia patients.[10] Until now, no studies have assessed ERFE in βTT patients referred for IO. Nevertheless, a population study in βTT Sri Lankan children found mild hepcidin suppression that was attributed to increased erythropoietic activity.[13] Similar studies in βTT adults[14] and children[15] showed a slight increase in hepcidin levels compared to healthy controls but a reduced hepcidin-to-ferritin ratio, in line with our results mentioned above. Of note, in our case series, ERFE was positively correlated with erythropoietin ($r=0.69$; $p=0.002$) and negatively correlated with hepcidin ($r=-0.54$; $p=0.020$), supporting the role of IE as a contributor to the relative deficiency of hepcidin in βTT.

The data collected in our observational case series at a tertiary center are consistent with those previously showing a subtle alteration of iron metabolism in βTT individuals, characterized by inappropriate hepcidin production in response to increasing iron stores, at least partly related to increased levels of the hepcidin inhibitor ERFE. Various cofactors, especially dysmetabolic features and alcohol consumption, can exacerbate this phenomenon in βTT individuals (as illustrated in Figure 1), leading to a clinically relevant IO that needs to be adequately recognized and treated. No guidelines are currently available for the management of iron overload in βTT individuals. Indeed, this "orphan" condition stands in the middle between classical hemochromatosis (generally treated with intensive phlebotomy) and IO in βT major or intermedia (where iron chelators are formally approved). We pragmatically offered our patients a personalized low-intensity venesection approach based on "mini-phlebotomies" (150-350 mL) every two to three weeks. The treatment was performed at our hospital, and Hb levels and patient symptoms were closely monitored. All patients underwent such treatment until ferritin was normalized (<300 or 200 μg/L in males and females, respectively), achieved in 6 to 36 months, depending on the severity of IO, Hb levels, and the personalized venesection schedule. Overall, this approach was effective and well tolerated, with no exacerbation of anemia. Rather, after the removal of excess iron, we sometimes observed a slight increase in Hb values compared to baseline. This finding is consistent with observations in a mouse model of myelodysplastic anemia, where iron chelation was associated with improved erythropoiesis.[16] This is possibly explained by the toxic effect of excessive iron on erythroid precursors in the bone marrow.

Figure 2 depicts a possible algorithm for the diagnosis of IO, starting from hyperferritinemia, that can be applied to all patients, while the management based on mini-phlebotomies should be reserved for mildly anemic βTT.

To the best of our knowledge, this is the first case series reporting a comprehensive pathophysiological evaluation and the feasibility of low-intensity venesection in patients with βTT and iron overload. Given the multifactorial pathophysiology, lifestyle improvement should be recommended in these patients, particularly aiming at alcohol avoidance and control of dysmetabolic features. A pragmatic and personalized approach and a strict follow-up at an expert center can effectively manage iron overload in mildly anemic βTT subjects. Further studies are warranted to corroborate our suggested approach.

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ID	Sex/ Age (years)	Main hematological parameters (Hb g/L; MCV fL; RBCs mil/mmc)	Other data supporting βTT diagnosis	Ferritin (µg/L) and TSat (%)	LIC at MRI (µmol/g)	Liver biopsy (Perl's stain)	HFE genotype	Daily alcohol intake (units)	Met S features	Cofactors for iron overload or hepcidin suppression	Hep (nM)	Hep-to-ferritin ratio (pmol/µg)	ERFE (ng/mL) and EPO (mUI/mL)
1	F/56	110; 61; 5.62	family history	700; 48	200	3+; fatty liver hepatitis and fibrosis	H63D +/-	0	D, IFG		6.9	9.9	37.8; 15.2
2	M/57	129; 68; 5.89	Codon 39 C>T	611; 55	n.a.	2+; fatty liver and cirrhosis	H63D +/-	8	None		7.2	11.8	9.4; 12
3	M/50	105; 60; 5.31	HbA_2 5.8%; HbF 1.7%	2,542; 100	>350	n.a.	H63D +/-	8	D		33.0	13.0	9.8; 6.6
4	M/70	110; 65; 5.34	family history	1,378; 100	n.a.	4+; mild fatty liver hepatitis	wild type	4	None		4.7	3.4	48.5; 26.9
5	M/57	110; 69; 5.49	HbA_2 5.1%; HbF 4.7%	1,334; 46	170	n.a.	wild type	8	O, U		14.8	11.1	39.3; 6.9
6	M/46	112; 61; 5.75	family history	880; 45	270	3+; mild hepatitis and fibrosis	H63D +/-	No	None		12.4	14.1	14.8; 16.9
7	M/75	120; 73; 5.50	family history	1,384; 58	250	n.a.	wild type	16	None		28.0	20.2	30.4; 20.9
8	F/67	115; 62; 6.12	family history	3,015; 70	230	n.a.	wild type	No	None	Prolonged IV IRT	20.2	6.7	8.8; 18.3
9	M/48	118; 71; 5.31	IVS 2.745 C>G	3,087; 98	290	n.a.	wild type	12	None	C virus hepatitis	6.1	2.0	73.5; 26.6
10	F/55	108; 62; 5.86	Codon 39 C>T	699; 48	300	3+; fatty liver and fibrosis	wild type	No	None		7.0	10.0	38.0; 14.9
11	M/46	100; 67; 5.42	Codon 39 C>T	935; 100	150	3+; severe fatty liver hepatitis and fibrosis	wild type	No	D, U		6.4	6.8	267.0; 45.9
12	M/64	123; 74; 5.20	Codon 39 C>T	847; 53	270	3+; fatty liver and cirrhosis	H63D +/+	12	None		11.8	13.9	40.1; 18.5
13	F/69	100; 70; 5.01	Codon 39 C>T	669; 77	n.a.	2+; cirrhosis	H63D +/-	No	None	C virus hepatitis	1.9	2.8	225.7; 43.3
14	M/64	125; 71; 5.65	Codon 39 C>T	619; 52	170	n.a.	wild type	16	O	Carrier of the *p.Glu112Gln* variant in the *BMP6* gene	13.0	21.0	44.4; 25.4
15	F/52	103; 70; 5.28	family history	932; 28	150	n.a.	wild type	No	None	Previous RBCs transfusion (30 units) for acute leukemia	23.9	25.6	60.6; 19.2
16	F/64	108; 64; 5.25	Codon 39 C>T	1,388; 65	n.a.	n.a.	C282Y/ H63D	No	None	Prolonged IV IRT	14.9	10.7	16.2; 14.8
17	M/64	129; 64; 6.08	HbA_2 6%; HbF 3.7%	715; 40	95	n.a.	wild type	No	H, O		15.3	21.4	29.3; 17.0
18	M/67	115; 63; 6.07	family history	1,636; 32	90	n.a.	H63D +/-	No	H, D, DM				
19	M/71	120; 68; 5.58	family history	1,302; 36	100	n.a.	wild type	4	O		12.0	9.2	14.9; 22.0
20	M/80	121; 69; 5.70	Codon 39 C>T	807; 66	200	n.a.	wild type	4	IFG				
21	M/70	110; 69; 5.15	IVS 1.1 G>A	2,642; 91	210	n.a.	wild type	No	None		22.8	8,6	
22	M/74	107; 74; 5.12	family history	994; 54	n.a.	3+; severe fatty liver	H63D +/-	8	H, D, DM				
23	M/72	116; 66; 5.53	family history	630; 80	3.23 ms*	n.a.	H63D +/+	No	H		11.2	17.8	
24	M/41	131; 66; 6.30	family history	762; 54	4.67 ms*	n.a.	wild type	No	O, D		24.5	32.2	
25	M/73	125; 62; 6.48	family history	1,060; 41	150	2+; mild fatty liver hepatitis	wild type	4	O				
26	M/57	121; 61; 6.29	Codon 39 C>T	1,081; 85	323	n.a.	C282Y +/+	No	None		2.87	2.7	
27	F/64	114; 63; 6.15	Codon 39 C>T	447; 70	n.a.	n.a.	C282Y +/+	No	D				
28	M/60	135; 67; 6.40	IVS 1-nt110 G>A	3,650; 87	n.a.	n.a.	C282Y +/+	No	H	Carrier of the *p.Gly895Ala* and *p.Cys534Trp* variants in the *CP* gene causing reduced CP levels (by ~50%)	0.5	0.1	
29	F/46	110; 66; 5.58	family history	994; 88	n.a.	4+; mild fibrosis	C282Y	No	None		3.6	3.6	

30	M/56	135; 69; 5.96	family history	3,100; 78	n.a.	3+; cirrhosis	+/+ C282Y +/+	2	H, O		1.4	0.5	

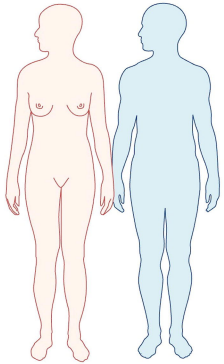
Table 1: Main hematological, biochemical, genetic, clinical data, and liver iron concentration (LIC) assessed by magnetic resonance imaging (MRI) and/or liver biopsy in our cohort of β-thalassemia trait (βTT) patients.

F: female; M: male. RBCs: red blood cells. MCV: mean corpuscular volume. TSat: transferrin saturation. n.a.: not available. Met S: metabolic syndrome. D: dyslipidemia; O: obesity; H: hypertension; IFG: impaired fast glucose; DM: diabetes mellitus; U: hyperuricemia. IV IRT: intravenous iron replacement therapy. CP: ceruloplasmin. Hep: hepcidin. ERFE: erythroferrone. EPO: erythropoietin.
Thirteen patients were genotyped for the *HBB* gene, revealing the following heterozygous variants: Codon 39 C>T, IVS 2.745 C>G, IVS 1.1 G>A, and IVS 1-nt110 G>A. No patients were genotyped for the *HBA* gene.
LIC by MRI was evaluated using Gandon's protocol in all subjects except for ID22 and ID23, who were studied using the MRI-T2 approach.
Normal reference ranges and values: MCV: 80-100 fL; RBCs: 4.00-5.20 mil/mmc; Hb: 120-150 g/L in females, 130-170 g/L in males; HbA_2 range for βTT: 3.55-5.85; Ferritin: 30-200 µg/L in women of reproductive age and 30-300 µg/L in men and post-menopausal women; TSat: 20-50%; normal LIC at MRI: <60 µmol/l (according to Gandon's protocol) or >6 ms (according to T2* approach); ERFE: 4-15 ng/ml; EPO: 1.6-34 mUI/mL.
° Age and sex-specific reference range of hepcidin and hepcidin-to-ferritin ratio were obtained in a sample of > 1,600 subjects evaluated in the framework of the "Valborbera study", described by Traglia M et al., 2011; in particular: hepcidin in females aged 18-29: 0.6-1.1; 30-39: 0.6-1.4; 40-49: 0.7-1.6; 50-59: 1.6-3.1; 60-69: 1.9-4.5; >70: 2.1-5.7; hepcidin in males aged 18-29: 2.4-3.3; 30-39: 2.3-3.6; 40-49: 3.4-4.9; 50-59: 2.8-4.4; 60-69: 2.3-4.4; >70: 1.9-4.7.

Figure 1: Factors and mechanisms contributing to iron overload in our cohort of β-thalassemia trait subjects.

Figure 2: Proposed flow-chart for the diagnosis and management of β-thalassemia trait patients with iron overload, starting from hyperferritinemia.

βTT subjects with IO
(n=30)



27% 73%

High alcohol consumption

H63D variant in the HFE gene

Advanced liver disease
(including hepatitis C virus, MAFLD, liver fibrosis, and cirrhosis)

Metabolic dysfunctions

Ineffective erythropoiesis
with increased ERFE

Hepcidin deficiency

Ferroportin

↑↑ liver iron

Ferroportin

↑↑ iron release from spleen macrophages recycling senescent erythrocytes

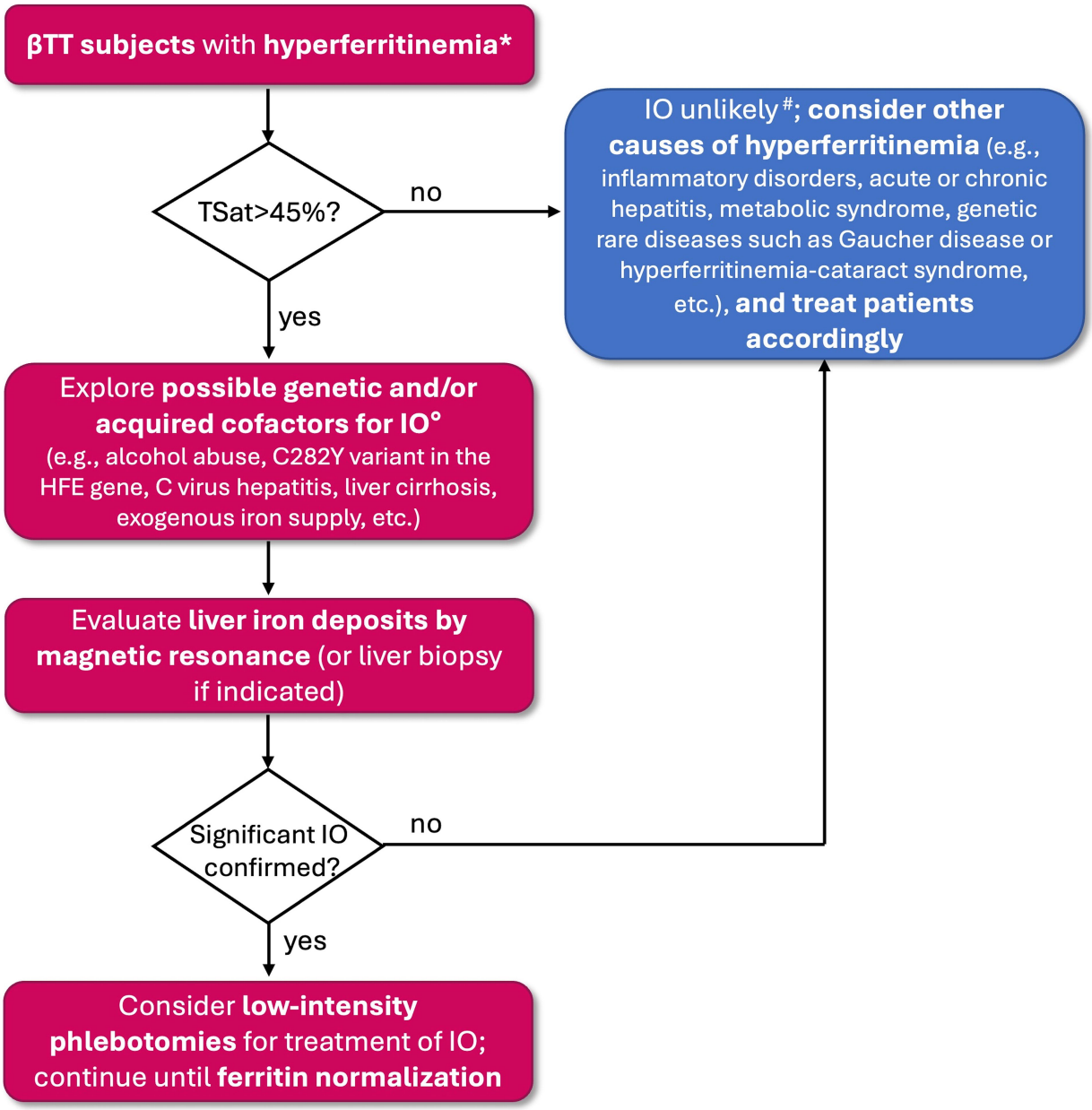
Ferroportin

↑↑ iron absorption

↑↑ plasma iron pool

Exogenous iron supply

MAFLD: metabolic-dysfunction associated fatty liver disease; ERFE: erythroferrone



* ferritin > 300 μg/L in males and post-menopausal women, > 200 μg/L in fertile women

° of note that subjects with true iron overload (IO) may present with concomitant other conditions possibly contributing to hyperferritinemia

rarely, IO may be present even if transferrin saturation (TSat) is normal-to-low (e.g., ferroportin disease, aceruloplasminemia)