



Liver iron concentrations and urinary hepcidin in β -thalassemia

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ABSTRACT

Background and Objectives

Patients with β -thalassemia, like those with genetic hemochromatosis, develop iron overload due to increased iron absorption, and their iron burden is further exacerbated by transfusion therapy. Heparin, a hepatic hormone, regulates systemic iron homeostasis by inhibiting the absorption of iron from the diet and the recycling of iron by macrophages. In turn, hepcidin release is increased by iron loading and inhibited by erythropoietic activity. Heparin deficiency is the cause of iron overload in most forms of hereditary hemochromatosis. We sought to determine hepcidin's role in the pathogenesis of iron overload in β -thalassemia.

Design and Methods

We assessed the degree of iron overload in thalassemia intermedia and major patients by measuring hepatic iron concentration in liver biopsy samples and serum ferritin, estimated erythropoietic drive by assaying soluble transferrin receptor and serum erythropoietin levels and correlated these with urinary hepcidin measurements.

Results

Urinary hepcidin levels in β -thalassemia demonstrate severe hepcidin deficiency in thalassemia intermedia. There was a strong inverse relationship between urinary hepcidin levels and both erythropoietin and soluble transferrin receptor, markers of erythropoietic activity. In contrast, hepcidin levels were elevated in thalassemia major, presumably due to transfusions that reduce erythropoietic drive and deliver a large iron load. Despite similar liver iron concentrations in the two conditions, serum ferritin was much lower in thalassemia intermedia.

Interpretation and Conclusions

In thalassemia intermedia, high erythropoietic drive causes severe hepcidin deficiency. The lack of hepcidin results in hyperabsorption of dietary iron, but also in iron depletion of macrophages, lowering their secretion of ferritin and, consequently, serum ferritin levels. In contrast, in thalassemia major, transfusions decrease erythropoietic drive and increase the iron load, resulting in relatively higher hepcidin levels. In the presence of higher hepcidin levels, dietary iron absorption is moderated and macrophages retain iron, contributing to higher serum ferritin. In the future, hepcidin measurements may allow a more accurate assessment of the degree of iron overload and the maldistribution of iron in thalassemia.

Key words: ineffective erythropoiesis, iron absorption, hepatic iron, iron-loading anemia.

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Complications from iron overload represent a major cause of morbidity and mortality in β -thalassemia. The iron loading in patients with thalassemia intermedia occurs more slowly than that in patients with thalassemia major, and is mainly due to increased intestinal absorption secondary to chronic anemia and ineffective erythropoiesis.^{1,2} Depending on the degree of the bone marrow expansion and peripheral hemolysis, the enhanced iron absorption accounts for the accumulation of about 2 to 5 grams of iron per year.¹ In thalassemia major, iron loading is accelerated by regular transfusions which contribute around 6 to 10 grams of iron per year.³ Although the rate of iron accumulation differs, the clinical consequences of iron loading are apparent in both groups of thalassemic patients and include liver, heart and endocrine abnormalities.^{4,5}

The increased iron absorption in thalassemia intermedia may be due to a deficiency of the iron-regulatory hormone hepcidin.⁶⁻⁹ Hepcidin deficiency, observed in most forms of hereditary hemochromatosis,¹⁰ is the key factor allowing excessive iron absorption and development of iron overload in these disorders. Hepcidin is a peptide produced by the liver in response to iron loading, but its synthesis is suppressed by anemia and hypoxia through an as yet unknown mechanism. We report here significantly lower hepcidin levels in thalassemia intermedia patients than in thalassemia major patients or controls, despite elevated iron parameters, indicating a dominant suppressive effect of increased erythropoiesis on hepcidin regulation in thalassemia intermedia. Estimation of iron overload in thalassemia has relied on serum ferritin measurements despite evidence that these do not always correlate well with liver iron concentration. In this study, we analyzed the relationship of serum ferritin to liver iron concentration in the context of the different pathophysiology of iron overload in thalassemia intermedia and major.

Design and Methods

All patient-related procedures were approved by the hospital Ethical Review Boards. Between 1995 and 2003, needle liver biopsy was performed in 22 patients with thalassemia intermedia, β^0 homozygotes for codon 39 G→A nonsense mutation and with normal alanine aminotransferase values. Fourteen of them had never been transfused and eight had received only sporadic transfusions during infections or surgery (less than ten blood units in total). Their mean hemoglobin level was 8.8 ± 1.1 g/dL and, due to the β gene molecular defect, their hemoglobin pattern was characterized by more than 95% of HbF, the rest of the hemoglobin being type HbA₂. For comparison, we considered 22 patients with thalassemia major, matched by sex, age and liver iron concentration, who had received regular transfusion therapy since childhood (mean annual Hb 11.3 ± 0.3 g/dL) and chelation with subcutaneous desferrioxamine 5 to 6 days per week, 8 to 12 hours per day.

Due to the regular transfusion therapy in these patients, their hemoglobin consisted mostly of HbA (>85%). These patients underwent liver biopsy for different reasons (chronic hepatitis, enrollment in clinical trials, preparation for bone marrow transplantation). The patients' characteristics are summarized in Table 1. Hepatic iron was determined by atomic absorption spectroscopy in liver biopsy samples and expressed in mg/g dry weight (d.w.) and serum ferritin (mean of at least two determinations performed within 2 months of the liver biopsy for each patient) by an automated chemiluminescence immunoassay analyzer (IMMULITE 2000®). Biopsy slides from 20 patients with thalassemia intermedia and 21 with thalassemia major were also evaluated for hemosiderin distribution in hepatocytes, K upffer cells and portal spaces after staining with Perls' Prussian blue according to Sciot semiquantitative iron scoring (scores ranging from 0 to 4) and for fibrosis after Goldner and Van Gieson's stain according to Desmet (scores ranging from 0 to 4).^{11,12} Serum erythropoietin, determined by chemiluminescence (Epo, Medical Systems, Genova, Italy), was evaluated in 12 patients with thalassemia intermedia and in 16 with thalassemia major. Transferrin receptor (s-TfR), determined by an immunocolorimetric method (TfR-c, Ramco Laboratories, Stafford, Texas, USA), was assessed in 16 patients with thalassemia intermedia and in 15 with thalassemia major. Urinary hepcidin levels could only be measured in a subgroup of ten patients with thalassemia intermedia and 11 patients with thalassemia major. Hepcidin values for each sample were determined at the University of California, Los Angeles using a previously described immunoassay.¹³ Hepcidin values were normalized to urinary creatinine concentration and urinary concentrations expressed as nanograms of hepcidin per milligram of creatinine.

Statistical analysis

Statistical comparison of quantitative variables was performed using the *t*-test for normally distributed variables and the Mann-Whitney test for variables that failed the normality test. One way ANOVA on ranks was used to compare hepcidin levels in thalassemia major, thalassemia intermedia and unrelated controls. Correlations between variables were evaluated by Spearman's correlation coefficient associated with a *t*-test. A multivariate analysis was performed to evaluate correlations between urinary hepcidin, serum ferritin, hemoglobin, s-TfR and serum erythropoietin. A type 1 error (α) of 0.05 was used to assess statistical significance in all tests.

Results

Liver iron concentration in patients with thalassemia intermedia was not significantly different from that in patients with thalassemia major, while the serum fer-

ritin levels were statistically significantly different between the two groups (Table 1).

Calculation of the Spearman's correlation coefficient showed that liver iron concentration was correlated with serum ferritin in thalassemia major patients ($r^2=0.46$, $p=0.001$) while no correlation was found in patients with thalassemia intermedia ($r^2=0.04$, $p=0.37$) (Figure 1).

In thalassemia intermedia patients, iron deposition was mainly found in the hepatocytes, with only occasional deposition in rare Kupffer cells (Figure 2A). In contrast, in liver biopsies from thalassemia major patients, we observed massive iron deposition in the majority of Kupffer cells, which appeared hypertrophic (Figure 2B). Only occasionally were fine hemosiderin granules detected in the cytoplasm of the hepatocytes (Figure 2B insert). The mean Sciot iron score in hepatocytes was 3.3 in patients with thalassemia intermedia and 2.9 in patients with thalassemia major ($p=0.05$), while in Kupffer cells it was 0.75 and 2.9, respectively, ($p<0.0001$) and in portal spaces 0.2 and 2, respectively ($p<0.0001$). In patients with thalassemia intermedia, fibrosis was absent in 17 samples, mild in two and moderate in one, whereas in patients with thalassemia major, fibrosis was mild in five, moderate in ten, severe in five, and one patient had cirrhosis.

As expected because of the expanded erythropoiesis, serum erythropoietin and s-TfR levels were significantly higher in patients with thalassemia intermedia than in those with thalassemia major [mean serum erythropoietin 426 ± 417 mU/mL (range 78-1720) vs 99 ± 121 (range 29-526 mU/ml) ($p<0.001$); mean s-TfR 43 ± 13 μ g/mL (range 25-80) vs 13 ± 6 μ g/mL (range 15-25) ($p<0.0001$)]. We determined urinary hepcidin concentrations in a subgroup of patients who were available for this part of the study. Their hematologic and iron

Table 1. Patients' characteristics including liver iron concentration and serum ferritin levels. Values are shown as mean \pm standard deviation.

	Thalassemia major	Thalassemia intermedia	p
Sex	11 M-11 F	14 M-8 F	0.5
Age (years)	23 ± 10	20 ± 5	0.08
HCV positive (%)	73	16	<0.0001
Spleen present (%)	100	14	<0.0001
Mean hemoglobin (g/dL)	11.3 ± 0.3	8.8 ± 1.1	<0.0001
LIC (mg/g d.w.)*	11.8 ± 7	11.3 ± 6	0.39
Ferritin (ng/mL)	2748 ± 2510	627 ± 309	0.0001

*LIC (liver iron concentration) normal range is 0.3–1.4 mg/g d.w.

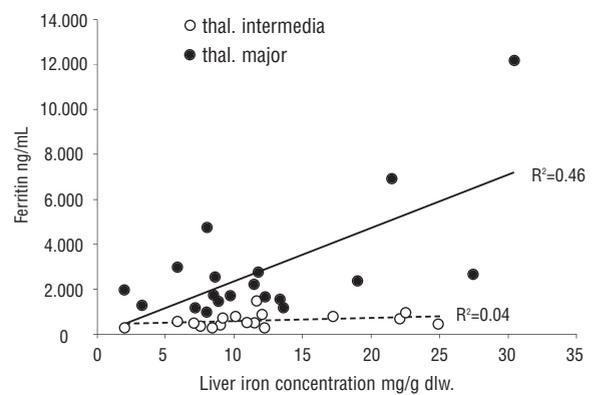


Figure 1. Correlation between liver iron concentration and serum ferritin in patients with β -thalassemia. The solid line represents the linear regression for thalassemia major ($n=22$), and the dashed line that for thalassemia intermedia ($n=22$).

parameters are summarized and statistically compared in Table 2. Urinary hepcidin concentration was below

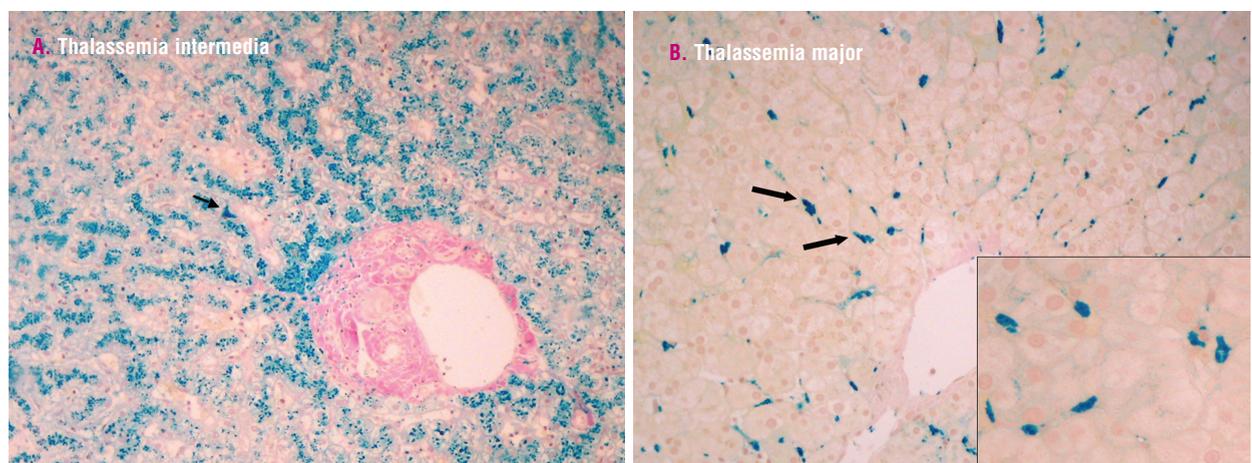


Figure 2. Iron distribution in the liver of thalassemia intermedia (A) and major (B) patients. A. Stored iron is found mainly at the biliary pole of hepatocytes. Only sporadic iron-loaded hypertrophic Kupffer cells are observed (arrow) (Perls' stain, original magnification is $\times 250$). B. Stored iron is observed mainly in hypertrophic Kupffer cells (arrows) (Perls' stain, original magnification is $\times 250$). At higher magnification ($\times 400$, insert), fine hemosiderin granules are also seen in the cytoplasm of hepatocytes.

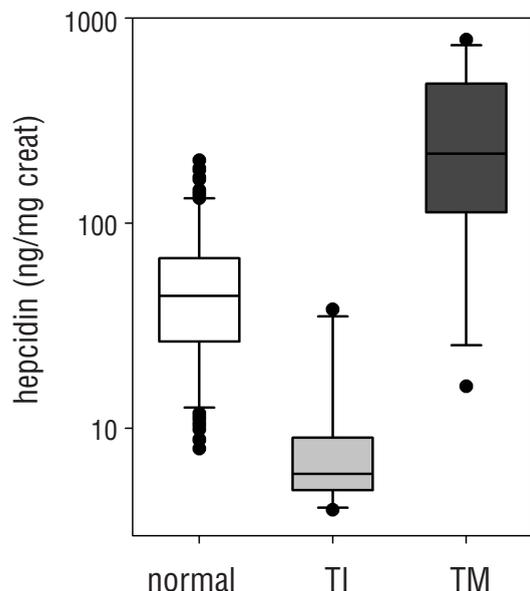


Figure 3. Urinary hepcidin levels in patients with thalassemia intermedia (TI, n=10), thalassemia major (TM, n=11) and unrelated control subjects (normal, n=105). The box-whisker plot shows the mean (horizontal line), 25-75% range (box), 5-95% range (whiskers), and values outside this range as closed circles. All three groups were significantly different from each other with $p < 0.05$ by one way ANOVA on ranks and Dunn's method.

the normal range in all but one patient with thalassemia intermedia, while it was in or above the normal range in all patients with thalassemia major (Figure 3). Hepcidin concentrations for 105 unrelated normal controls are provided for reference in Table 2.

The mean hepcidin-to-ferritin ratio was significantly lower in patients with thalassemia intermedia than in those with thalassemia major and was low in both groups of patients when compared to controls (Table 2).

Considering all patients, urinary hepcidin levels showed the strongest and inverse correlation with s-TfR ($r^2=0.83$, $p < 0.001$, Figure 4A). Hepcidin levels also correlated inversely with serum erythropoietin ($r^2=0.56$, $p < 0.001$, Figure 4B) and positively with serum ferritin ($r^2=0.68$, $p < 0.001$, Figure 4C) and hemoglobin ($r^2=0.42$, $p=0.002$, Figure 4D). Importantly, the level of hepcidin in patients with thalassemia intermedia was decreased despite high transferrin saturation (79 ± 22 %), which was not significantly different from transferrin saturation in patients with thalassemia major (91 ± 13 %, $p=0.072$).

Discussion

Iron overload in non-transfused thalassemia intermedia patients occurs because of increased intestinal iron absorption due to greatly expanded but ineffective erythropoiesis^{1,2} while in regularly transfused thalassemia major the overload is mostly due to red cell breakdown.

Table 2. Hematologic and iron parameters in a subgroup of patients in whom hepcidin was assayed. Values are shown as medians and full range.

	Thalassemia Major (n=11)	Thalassemia Intermedia (n=10)	p	Reference range
Hemoglobin (g/dL)	10.0 (9.5-11.3)	8.2 (6.8-10.3)	0.002	11-18 g/dL
Hepcidin (ng/mg creatinine)	218 (16-784)	6 (4-38)	<0.001	44 (10-200)
Ferritin (ng/mL)	3000 (1000-4480)	462 (303-1314)	<0.001	25-280 ng/mL
Hepcidin/ferritin ratio	0.10 (0.02-0.30)	0.01 (0.005-0.12)	0.001	0.24 (0.04-3.3)
sTfR (μ g/mL)	13 (5-20)	42 (37-80)	<0.001	< 8
EPO (mU/mL)	62* (29-197)	360* (116-1720)	<0.001	< 30

*One value missing; EPO: erythropoietin.

Although the rate of iron loading is slower in thalassemia intermedia than in thalassemia major, patients with thalassemia intermedia can eventually develop complications similar to those of patients with thalassemia major, including hepatic, endocrine and cardiac dysfunction.⁴ In contrast, hyperabsorption of iron is not a prominent feature of hemolytic anemias with efficient erythropoiesis, such as HbH disease. Assessment of iron overload in thalassemia is important for the patients' management. The most commonly used methods for assessing this overload include measurements of serum ferritin and liver iron concentration (LIC). While in patients with thalassemia major the correlation between these two parameters has been evaluated with variable results,^{14,15} in thalassemia intermedia, despite the notion that ferritin measurements do not accurately reflect the level of iron overload,¹⁶ the data correlating the two methods are still lacking. In this study, we found that the correlations of liver iron concentration and ferritin differed between patients with thalassemia major and those with thalassemia intermedia. Serum ferritin was significantly lower in patients with thalassemia intermedia than in those with thalassemia major, despite the liver iron concentration being comparable. Lower serum ferritin in thalassemia intermedia reflects iron accumulation predominantly in hepatocytes rather than in macrophages, as observed by Perls' stain. This phenotype is similar to that occurring in hereditary hemochromatosis and is likely the consequence of hepcidin deficiency. As in previous studies,^{7,8} we also found very low or undetectable hepcidin levels in thalassemia intermedia. Hepcidin, a 25-amino acid peptide synthesized by hepatocytes, is the negative regulator of iron absorption in the small intestine and iron release from macrophages recycling red blood cells.⁶ Hepcidin deficiency likely allows greater export of iron

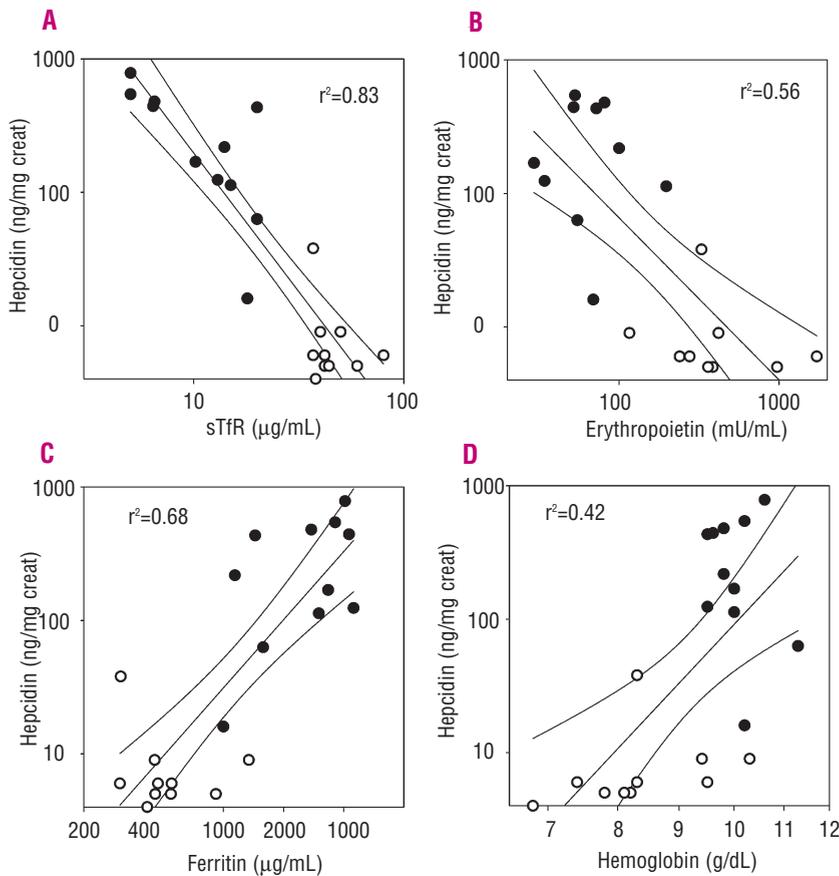


Figure 4. Correlations of urinary hepcidin with indices of erythropoiesis and iron loading in β -thalassemia. Graphs show linear regression with the 95% confidence interval for correlations of urinary hepcidin with sTfR (A), erythropoietin (B), ferritin (C) and hemoglobin (D).

from macrophages, thus lowering macrophage cytoplasmic iron and suppressing secretion of soluble ferritin. Additionally, it results in increased iron absorption, oversaturation of transferrin and accumulation of non-transferrin-bound iron leading to predominantly parenchymal iron overload.

Hepcidin synthesis is stimulated by iron loading and suppressed by anemia,^{17,18} and in thalassemia syndromes, both of these opposing factors would be expected to regulate hepcidin production. The pathway of hepcidin regulation by anemia is still unclear, but recent studies suggest that it involves an erythropoietic signal¹⁹ or changes in iron levels as it is being consumed for erythropoiesis.²⁰ Since our patients with thalassemia intermedia not only had expanded erythropoiesis as reflected by their sTfR and serum erythropoietin levels, but also elevated transferrin saturation and iron stores, the finding of hepcidin deficiency in these patients supports the existence of a suppressive erythropoiesis-related factor which is distinct from and dominant over the iron signal. This is in agreement with the mouse models with concurrent iron overload and severe anemia caused by phenylhydrazine treatment,¹⁷ hypotransferrinemia²¹ or thalassemia trait (Hbb^{th5/+}),⁹ which also showed decreased hepcidin expression.

Hepcidin levels were higher in thalassemia major patients than in thalassemia intermedia patients or normal controls. The difference in hepcidin levels between

thalassemia intermedia and major is almost certainly due to transfusion therapy. Transfusions suppress the erythropoietic drive and increase body iron load, both of which would result in an increase of hepcidin. Indeed, Kearney *et al.* measured urinary hepcidin before and 3-4 days after transfusion, and showed that most patients responded by increasing hepcidin levels.⁸

By causing iron retention in macrophages,^{22,23} elevated hepcidin levels in thalassemia major may also contribute to the phenotype of iron accumulation in K pffer cells which we observed on histological analysis. Although experimental evidence is lacking, macrophages are thought to contribute significantly to serum ferritin.²⁴ Iron accumulation in macrophages could raise serum ferritin levels by increasing ferritin synthesis via the IRE/IRP system. However, when the ratio of urinary hepcidin to serum ferritin was analyzed as an index of appropriateness of hepcidin response to iron load,^{8,25} the ratio was low in both thalassemia syndromes when compared to controls. Thus, even in patients with thalassemia major, hepcidin is inappropriately low relative to the patients' iron load, indicating that transfusions only partially relieve the erythropoietic drive.

Hypoxia was shown to suppress hepcidin directly in hepatocyte cell lines.¹⁷ Because patients with thalassemia intermedia had lower oxygen delivery to tissues due to lower hemoglobin concentration and a high

proportion of fetal hemoglobin, some contribution of hypoxia to greater hepcidin suppression in these patients cannot be excluded. Potential additional confounding factors include higher frequency of splenectomies in patients with thalassemia intermedia and higher rates of hepatitis C virus (HCV) infection in those with thalassemia major. In a previous study, however, splenectomies did not significantly influence hepcidin levels.⁸ It is also unlikely that inflammation related to active HCV infection contributed to increased hepcidin in patients with thalassemia major because all such patients had normal alanine aminotransferase levels and seven out of nine anti-HCV positive patients in whom hepcidin was assayed were HCV RNA negative, one was positive and in one HCV RNA was not determined.

In conclusion, we suggest that in thalassemia intermedia, an as yet unknown signal from expanded erythropoiesis strongly suppresses hepcidin production, resulting in increased intestinal iron absorption and eventual parenchymal iron overload. The observation that serum

ferritin levels underestimate the severity of iron overload has important implications for iron chelation in patients with thalassemia intermedia. In such patients, serum ferritin levels have little value for the monitoring of iron overload and iron chelation, and alternative assessments such as liver iron concentration by biopsy, superconducting quantum-interference device (SQUID) or magnetic resonance imaging should be used. Moreover, hepcidin measurements may be useful as part of the diagnostic and prognostic evaluation of thalassemia syndromes. In the future, it may be possible to use exogenous hepcidin to restore normal iron homeostasis in patients with thalassemia intermedia.

Authors' Contributions

RO, RG and TG designed the research, analyzed the data and wrote the paper; NG and LM performed the research and contributed to the data analysis; GF analyzed patients' histological samples; EN designed and performed the research, analyzed the data and wrote the paper.

Conflict of Interest

The authors reported no potential conflicts of interest.

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