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Serum ferritin and transferrin saturation levels in β^0 and β^+ thalassemia patients

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ABSTRACT. There have been few studies on the mutations that cause heterozygous beta-thalassemia and how they affect the iron profile. One hundred and thirty-eight individuals were analyzed, 90 thalasemic β^0 and 48 thalasemic β^+, identified by classical and molecular methods. Mutations in the hemochromatosis (*HFE*) gene, detected using PCR-RFLP, were found in 30.4% of these beta-thalassemic patients; heterozygosity for H63D (20.3%) was the most frequent. Ferritin levels and transferrin saturation were similar in beta-thalassemics with and without mutations in the *HFE* gene. Ferritin concentrations were significantly higher in men and in individuals over 40 years of age. Transferrin saturation also was significantly higher in men, but only in those without *HFE* gene mutations. There was no significant difference in the iron profile among the β^0 and β^+ thalassemics, with and without *HFE* gene mutations. The frequency of ferritin values above 200 ng/mL in women and 300 ng/mL in men was also similar in β^0 and β^+ thalassemics ($P > 0.72$). Our conclusion is that ferritin levels are variable in the beta-thalassemia, trait regardless of the type of beta-globin mutation. Furthermore, *HFE* gene polymorphisms do not change the iron profile in these individuals.

Key words: Ferritin; Beta-thalassemia; Hyperferritinemia; Transferrin saturation

INTRODUCTION

Ferritin is the main iron-storage protein in the body. Its synthesis is regulated by quantities of iron by means of the interaction of cytoplasmic proteins bound to the messenger ribonucleic acid (mRNA), currently identified as iron regulatory proteins with specific structures of the mRNA, called iron-responsive elements (Kannengiesser et al., 2009). It has a central role in iron homeostasis, because it binds to and sequesters intracellular iron. Serum ferritin measurement has become a routine laboratory test and high levels are a common finding in clinical practice. High serum ferritin levels are found in a large spectrum of genetic and acquired conditions, whether associated or not with iron overload. The precise diagnosis of hyperferritinemia is difficult and requires a detailed medical history, blood biochemistry and sometimes, genetic tests. An increase in ferritin levels combined with an increase in transferrin saturation usually is a sign of iron overload (Camaschella, 2005; Brissot et al., 2008; Chamaschella and Poggiali, 2009).

The molecular basis of beta-thalassemia has been largely elucidated. The reported mutations affect all the known stages of beta-globin gene expression, resulting in a decrease (β^+) or complete absence (β^0) of beta-globin synthesis of the affected alleles (Weatherall, 2007). Beta-thalassemia major is clinically characterized by transfusion-dependent severe anemia. A mild course of the illness is observed in beta-thalassemia intermedia and a clinically discreet phenotype is common in the beta-thalassemia trait.

Hemochromatosis is frequently observed in beta-thalassemia major due to an increased rate of iron absorption by the gastrointestinal tract and frequent blood transfusions. In the beta-thalassemia trait, there is some degree of ineffective erythropoiesis, which leads to heightened erythropoietic activity and increased iron absorption (Demir et al., 2004). However, only a minority of patients with the beta-thalassemia trait develop iron overload, indicating that other factors are involved in these cases (Fargion et al., 1985).

Excess iron is extremely toxic to all cells of the body and can cause serious and irreversible organic damage, such as cirrhosis, diabetes, heart disease, and hypogonadism (Melchiori et al., 2010). High levels of serum ferritin have been observed in beta-thalassemia trait comparative studies, and even those who had never been transfused developed clinical and laboratory signs of iron overload (Edwards et al., 1981; Fargion et al., 1982, 1985; Piperno et al., 2000). The pathophysiology of this complication associated with heterozygous thalassemia is unclear. Several researchers have suggested a modulating effect of the beta-globin gene mutation and proteins related to iron metabolism (Fargion et al., 1985; Piperno et al., 2000; Melis et al, 2002; Martins et al., 2004). Individuals with ferritin levels above 300 ng/mL in men and 200 ng/mL in women and/or transferrin saturation above 45% who show no signs of inflammatory disease must be investigated for iron overload (Camaschella, 2005; Yen et al., 2006).

The aim of this study was to evaluate the influence of β^0 and β^+ mutations on serum ferritin levels and transferrin saturation and correlate this with the phenotypic manifestations and co-heritage with *HFE* gene mutations in carriers of the beta-thalassemia trait.

MATERIAL AND METHODS

In this study, 138 individuals were analyzed, 37.7% men and 62.3% women, between 20 and 90 years of age, with the beta-thalassemia trait identified through clinical evaluation and laboratory tests, which included the Bio-Rad VARIANT HPLC automated testing system

with the "β Thalassemia Short Program" kit and allele-specific polymerase chain reaction (AS-PCR) molecular analysis in order to identify β^0 (CD 39) and β^+ (IVS 1-110 and IVS 1-6) mutants (Bertholo and Moreira, 2006).

Transferrin saturation was calculated by dividing the serum iron level by total iron-binding capacity and multiplied by 100. Serum iron levels as well as the iron-binding capacity levels were obtained through the two-point enzymatic method using VITROS Chemistry Systems (Ortho-Clinical Diagnostics Products, Johnson & Johnson, USA) and ferritin levels were obtained through direct chemiluminometric technology, using the ADVIA Centaur® system. All thalassemics were submitted to the C-reactive protein test using the immunoturbidimetry method.

The upper limits for normal ferritin levels were 200 and 300 ng/mL in women and men, respectively. For transferrin saturation, levels above 45% were considered to be elevated (Carmaschella, 2005; Brissot et al., 2008). The presence of the *HFE* gene mutation was investigated using the PCR-RFLP method (Lynas, 1997).

The study was approved by the UNESP Ethical Committee and informed consent was obtained before the sample was collected.

Statistical analysis

Statistical analysis was obtained by the Minitab 14 Statistical software. The methods used were parametric for transferrin saturation (Student *t*-test) and non-parametric for ferritin (Mann-Whitney test). The incidences for the *HFE* gene polymorphisms in individuals with ferritin levels above those considered to be normal and transferrin saturation above 45% were compared by means of the Fisher exact test. The level of significance for all tests was considered to be $P < 0.05$.

RESULTS

Ninety (65.2%) individuals with the CD 39 mutation were identified, 39 (28.3%) with the IVS 1-110 mutation and 9 (4.3%) with the IVS 1-6 mutation. Among the 138 individuals evaluated, 42 (30.4%) also presented mutations in the *HFE* gene and the most frequent genotype found was heterozygous for the H63D mutation (20.3%).

Among the carriers of the CD 39 mutation, 28 (31.1%) presented mutations in the *HFE* gene, 24 (85.7%) had H63D polymorphism, 17 heterozygous, 4 homozygous and 3 double heterozygous, C282Y/H63D. Among the IVS 1-110 mutation carriers, 14 (35.9%) also presented mutation for the *HFE* gene, 11 (78.6%) heterozygous for H63D. No polymorphism for the *HFE* gene was found in IVS 1-6 thalassemics. In the evaluation of *HFE* gene polymorphisms, no cases of homozygosity for C282Y or S65C mutation were detected. The results of this molecular characterization are summarized in Table 1.

Table 1. Presence of mutations that determine heterozygous beta-thalassemia and *HFE* gene polymorphisms.

Mutations	H63D (+ / +) (N = 4)	H63D (+ / -) (N = 28)	C282Y (+ / -) (N = 6)	C282Y/H63D (N = 4)	Absence of mutation in the *HFE* gene (N = 96)
CD 39 N = 90 (65.2%)	4 (4.4%)	17 (18.9%)	4 (4.4%)	3 (3.3%)	62 (68.9%)
IVS 1-110 and IVS 1-6 N = 48 (28.3%)	0	11 (22.9%)	2 (4.2%)	1 (2.1%)	34 (70.8%)

Ferritin concentration in the beta-thalassemia trait with polymorphism in the *HFE* gene varied from 6.2 to 2982.0 ng/mL and transferrin saturation, from 6.5 to 77.2%. In thalassemics without mutations in the *HFE* gene, ferritin levels varied from 10.0 to 1420.0 ng/mL and transferrin saturation, from 12.3 to 84.8%. There was no significant difference between ferritin and transferrin saturation averages among beta-thalassemia carriers with and without polymorphism in the *HFE* gene. These results are detailed in Table 2.

Table 2. Ferritin[a] and transferrin saturation[b] in heterozygous beta-thalassemics with and without polymorphism of the *HFE* gene.

Gender and age	Polymorphisms in the *HFE* gene	Ferritin (ng/mL)	P[c]	Transferrin saturation (%)	P[d]
Female	Present	25.7 to 226.0	0.18	21.3 to 53.1	0.20
18 to 40 (years)	(N = 10)	53.5		34.4 ± 9.0	
	Absent	10.0 to 234.0		14.1 to 49.5	
	(N = 32)	70.1		29.8 ± 10.2	
Female	Present	6.2 to 2982.0	0.82	6.5 to 67.8	0.25
>40 (years)	(N = 18)	127.0		30.0 ± 14.1	
	Absent	19.0 to 536.0		12.3 to 52.7	
	(N = 26)	128.5		25.3 ± 9.4	
Male	Present	58.0 to 268.0	0.09	25.4 to 44.0	0.31
18 to 40 (years)	(N = 6)	154.0		35.1 ± 8.0	
	Absent	26.0 to 1420.0		17.3 to 84.8	
	(N = 18)	233.0		40.8 ± 18.3	
Male	Present	38.3 to 1034.0	0.66	23.3 to 77.2	0.98
>40 (years)	(N = 8)	474.0		39.3 ± 17.1	
	Absent	45.0 to 1192.0		19.1 to 62.9	
	(N = 20)	268.0		39.1 ± 12.6	

[a]Data are reported as minimum, maximum and median. [b]Data are reported as minimum, maximum and means ± standard deviation. [c]Mann-Whitney test, significant at $P < 0.05$. [d]Student *t*-test, significant at $P < 0.05$.

In thalassemics with and without polymorphism in the *HFE* gene, ferritin values were higher in men than in women as well as in individuals over 40 years of age. Transferrin saturation also was higher in men, although only in those without polymorphism in the *HFE* gene (Table 3).

Table 3. Ferritin[a] and transferrin saturation[b] in heterozygous beta-thalassemics with or without polymorphism of the *HFE* gene in different genders and age groups.

Polymorphisms in the *HFE* gene	Gender and age	Ferritin (ng/mL)	P[c]	Transferrin saturation (%)	P[d]
Present	Male	38.3 to 1034.0	0.01	23.3 to 77.2	0.20
	(N = 14)	221.0		37.5 ± 13.7	
	Female	6.2 to 2982.0		6.5 to 67.8	
	(N = 28)	79.0		31.7 ± 12.4	
Absent	Male	26.0 to 1420.0	0.01	17.3 to 84.8	0.01
	(N = 38)	253.0		39.9 ± 15.3	
	Female	10.0 to 536.0		12.3 to 52.7	
	(N = 58)	87.5		27.8 ± 10.0	
Present	18 to 40 (years)	25.7 to 268.0	0.03	21.3 to 53.1	0.69
	(N = 16)	68.0		34.7 ± 8.4	
	>40 (years)	6.2 to 2982.0		6.5 to 77.2	
	(N = 26)	183.0		33.1 ± 15.4	
Absent	18 to 40 (years)	10.0 to 1420.0	0.01	14.1 to 84.8	0.38
	(N = 50)	100.3		33.7 ± 14.4	
	>40 (years)	19.0 to 1192.0		12.3 to 62.9	
	(N = 46)	181.9		31.2 ± 12.7	

[a]Data are reported as minimum, maximum and median. [b]Data are reported as minimum, maximum and means ± standard deviation. [c]Mann-Whitney test, significant at $P < 0.05$. [d]Student *t*-test, significant at $P < 0.05$.

In order to better evaluate the influence of beta-globin mutations, the samples were separated according to age and gender, and carriers of polymorphism in the *HFE* gene were excluded. There was no significant difference in ferritin concentrations and transferrin saturation in the evaluated groups for β^0 and β^+ thalassemics. The results are presented in Table 4.

Table 4. Influence of the mutation of the beta-globin gene, age and gender on ferritin[a] values and transferrin saturation[b] in heterozygous thalassemics without polymorphism in the *HFE* gene.

Gender and age	Beta-globin mutation	Ferritin (ng/mL)	P[c]	Transferrin saturation (%)	P[d]
Female	β^0 (N = 21)	10.0 to 134.0	0.38	14.1 to 49.1	0.19
18 to 40 (years)		69.2		28.1 ± 10.0	
	β^+ (N = 11)	34.0 to 234.0		19.0 to 49.5	
		71.0		33.2 ± 10.2	
Female	β^0 (N = 19)	34.9 to 536.0	0.06	12.3 to 40.1	0.40
>40 (years)		152.0		24.1 ± 8.3	
	β^+ (N = 7)	19.0 to 490.0		16.4 to 52.7	
		58.0		28.6 ± 12.1	
Male	β^0 (N = 9)	146.0 to 1420.0	0.33	21.5 to 84.8	0.84
18 to 40 (years)		342.0		41.7 ± 20.5	
	β^+ (N = 9)	26.0 to 576.0		17.3 to 73.1	
		222.0		39.8 ± 16.9	
Male	β^0 (N = 13)	85.0 to 1010.0	0.87	19.1 to 62.9	0.94
>40 (years)		262.0		39.3 ± 13.5	
	β^+ (N = 7)	45.0 to 1192.0		21.8 to 56.0	
		309.0		38.8 ± 11.7	

[a]Data are reported as minimum, maximum and median. [b]Data are reported as minimum, maximum and means ± standard deviation. [c]Mann-Whitney test, significant at $P < 0.05$. [d]Student *t*-test, significant at $P < 0.05$.

In those thalassemics with a concurrent presence of *HFE* gene polymorphisms, 11 (26.2%) had ferritin levels above those established as normal (eight β^0 and three β^+) and of these, only three (7.1%) had transferrin saturation above 45% (one β^0 and two β^+) (Table 5).

Table 5. Ferritin above the values established as normal and transferrin saturation above 45% in heterozygous beta-thalassemia β^0 and β^+, with and without polymorphism of the *HFE* gene.

Polymorphisms in the *HFE* gene	Thalassemia	Ferritin >300 ng/mL in men and 200 ng/mL in women	P*	Ferritin above normal levels and transferrin saturation >45%	P*
Present	β^0	8	0.72	1	0.25
N = 42	(N = 28)	(28.6%)		(3.6%)	
	β^+	3		2	
	(N = 14)	(21.4%)		(14.3%)	
Absent	β^0	17	1.00	5	1.00
N = 96	(N = 62)	(27.4%)		(8.1%)	
	β^+	9		2	
	(N = 34)	(26.5%)		(5.9%)	
Total	β^0	25	0.85	6	0.74
N = 138	(N = 90)	(27.8%)		(6.7%)	
	β^+	12		4	
	(N = 48)	(28.6%)		(8.3%)	

*Fisher exact test, significant at $P < 0.05$.

Of the four patients with double heterozygosity, C282Y/H63D, three were males and one female. All men had ferritin levels above 500 ng/mL; nonetheless, transferrin saturation above 45% was found in just one of those individuals. Ferritin concentrations above 1000 ng/mL were observed in one individual, whose transferrin saturation was below 45%. Of those heterozygous for C282Y, four were females and two males; two had ferritin levels above 45% (45.4 and 67.8%,

respectively). Homozygosity for H63D polymorphism was found in four women, and only one had ferritin values above those established as normal, although with normal transferrin saturation. Heterozygosity for H63D was found in 19 women and 9 men, 4 of them (10.7%) with ferritin levels above those established as normal, but all had transferrin saturation below 45%.

Of the 62 carriers of β^0 (CD 39) mutation and without the presence of polymorphism in the *HFE* gene, 17 (27.4%) had ferritin above the levels established as normal and of these, 8 (12.9%) had levels higher than 500 ng/mL. Transferrin saturation above 45% was observed in 5, all with ferritin levels above 500 ng/mL.

Among the carriers of the β^+ mutation (IVS 1-110 and IVS 1-6) without polymorphisms of the *HFE* gene, 9 (26.5%) had ferritin levels above those established as normal, and in 4, levels were above 500 ng/mL. Two of these thalassemics had transferrin saturation above 45%. Of the thalassemics without the *HFE* gene mutation, 27.1% had ferritin levels above those established as normal and 7.3% had a concurrent increase in transferrin saturation (>45%). Of these, five were β^0 mutants (CD 39) and two β^+ (IVS 1-110) (Table 5).

Two thalassemics with β^0 mutation (CD 39) and one with β^+ (IVS 1-6), all males, had ferritin values above 1000 ng/mL. Of these, only one β^0 had a concurrent increase in transferrin saturation (84.8%). Besides, the other two thalassemics presented comorbidities associated with the increase in ferritin concentration, such as diabetes mellitus and chronic alcoholism. No increase in C-reactive protein levels was detected in thalassemics with serum ferritin levels above those established as normal.

DISCUSSION

Among the beta-thalassemics evaluated, ferritin concentration presented a heterogeneous pattern and 27% had ferritin serum levels above those established as normal, with and without an increase in transferrin saturation. Beta-thalassemia syndromes are characterized by quantitative alterations in the synthesis of the beta-chains of hemoglobin. Excess alpha-chains precipitate in cellular membranes and result in variable levels of premature destruction of erythrocytes and their precursors, as well as ineffective erythropoiesis and anemia. No significant differences were observed in iron profile results among β^0 and β^+ thalassemics, suggesting that the absence or decrease in beta-globin synthesis, when in heterozygosity, influences iron absorption in these individuals in the same way.

In the severe forms of beta-thalassemia, multiple blood transfusions and a deficiency of hepcidin, a potent iron absorption inhibitor, result in iron overload with increased serritin serum concentration and transferrin saturation (Brissot et al., 2008). This accumulation results in clinical problems similar to those of primary hemochromatosis. In the milder forms, ineffective erythropoiesis is discreet and these individuals rarely present iron excess (Lewis et al., 1965; Parfrey et al., 1981; Garcia et al., 1993). Yet, in heterozygous β^0 thalassemics, a higher imbalance between the quantities of the different globin chains could justify the increase in iron absorption and, consequently, high levels of ferritin and transferrin saturation. However, our results do not corroborate that hypothesis.

High ferritin values have been described in heterozygous thalassemics with mutations in other genes related to iron homeostasis control (Fargion et al., 1985; Piperno et al., 2000; Melis et al., 2002).

C282Y and H63D mutations in the *HFE* gene, more commonly observed in individu-

als with hereditary hemochromatosis, are frequent in the northern region of Europe. In Italy, in spite of the smaller incidence of C282Y mutation, the prevalence of the H63D mutation is high (Restagno et al., 2000; Cassanelli et al., 2001; Girouard et al., 2002; Miniero et al., 2005). In that country, beta-thalassemia gene mutations are also frequent. Therefore, due to the influence of these individuals in the composition of the Brazilian population, the co-inheritance findings of the mutations of the beta-globin gene with the H63D mutation reinforce the incidence of this mutation among the evaluated β^0 and B^+ thalassemics.

Homozygosity for C282Y and double heterozygosity for C282Y and H63D are frequently associated with ferritin increase and transferrin saturation, but for other genotypes, these metabolic alterations are less frequent (Aranda et al., 2010). The iron profile of heterozygous thalassemics with or without mutations in the *HFE* gene was similar, but due to the high occurrence of heterozygosity for H63D, our results mainly reflect the influence of this polymorphism in the observed ferritin concentration and transferrin saturation.

Most times, serum ferritin levels are related to the quantity of iron stored in the body; however, countless other genetic and acquired conditions, with and without iron overload, can influence these results (Camaschella and Poggiali, 2009). An increase in ferritin concentrations with no excess iron body can be observed in acute or chronic inflammatory processes, autoimmune diseases, neoplasias, chronic renal insufficiency, hepatopathies, and metabolic syndrome. In these conditions, transferrin saturation generally is normal or decreased. On the other hand, when there is an iron overload, except in ferroportin disease, the increase in ferritin concentrations is associated with increased saturation of transferrin (Brissot et al., 2008; Camaschella and Poggiali, 2009).

In this study, only 27% of thalassemia carriers with ferritin levels above 300 ng/mL in men and 200 ng/mL in women had transferrin saturation above 45%, suggesting that in most cases, this increase was probably not associated exclusively with iron reserves. However, acute or chronic inflammatory diseases were discarded due to normal results in C-reactive protein. Even among the general population, the percentage of patients with high ferritin levels who have an increase in iron reserves is still unknown (Gordeuk et al., 2008).

Based on our results, we can conclude that ferritin concentrations have a heterogeneous pattern in heterozygous beta-thalassemia, and are higher in men than in women and in individuals over 40 years of age, stressing the importance in separating individuals according to gender and age group in evaluation studies for iron homeostasis. High levels of ferritin serum observed in heterozygous beta-thalassemics do not depend on the inherited mutation in the beta-globin gene and the association of heterozygous beta-thalassemia with polymorphism in the *HFE* gene, especially the H63D mutation in heterozygosity, does not modify the iron profile in these individuals. In most thalassemics with increased ferritin, levels rarely exceed 1000 ng/mL and, in general, there is no concurrent increase in transferrin saturation. However, in 7.2% of all heterozygous beta-thalassemics, we can observe increased ferritin levels and increased transferrin saturation, warranting that these individuals should be evaluated and monitored for diseases associated with iron overload.

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