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HFE gene mutations and iron status of Brazilian blood donors

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Abstract

Mutations of the *HFE* and *TFR2* genes have been associated with iron overload. *HFE* and *TFR2* mutations were assessed in blood donors, and the relationship with iron status was evaluated. Subjects (N = 542) were recruited at the Hemocentro da Santa Casa de São Paulo, São Paulo, Brazil. Iron status was not influenced by *HFE* mutations in women and was independent of blood donation frequency. In contrast, men carrying the *HFE* 282CY genotype had lower total iron-binding capacity (TIBC) than *HFE* 282CC genotype carriers. Men who donated blood for the first time and were carriers of the *HFE* 282CY genotype had higher transferrin saturation values and lower TIBC concentrations than those with the homozygous wild genotype for the *HFE* C282Y mutation. Moreover, in this group of blood donors, carriers of *HFE* 63DD plus 63HD genotypes had higher serum ferritin values than those with the homozygous wild genotype for *HFE* H63D mutation. Multiple linear regression analysis showed that *HFE* 282CY leads to a 17.21% increase (P = 0.018) and a 83.65% decrease (P = 0.007) in transferrin saturation and TIBC, respectively. In addition, serum ferritin is influenced by age (3.91%, P = 0.001) and the *HFE* 63HD plus DD genotype (55.84%, P = 0.021). In conclusion, the *HFE* 282Y and 65C alleles were rare, while the *HFE* 63D allele was frequent in Brazilian blood donors. The *HFE* C282Y and H63D mutations were associated with alterations in iron status in blood donors in a gender-dependent manner.

Key words: HFE; TFR2; Gene mutations; Blood donors; Iron status

Introduction

Iron is essential for the adequate functioning of metabolic and structural proteins in cells. Proteins, such as HFE, hemojuvelin, transferrin receptor 2 (TFR2), and ferroportin, and a peptide called hepcidin, regulate iron metabolism. Mutations in the genes of these proteins or peptide are associated with the etiology of iron overload (hereditary hemochromatosis, HH), which is characterized by increased intestinal iron absorption and progressive accumulation of iron in the body (1).

The HFE protein forms a complex with β_2-microglobulin and this complex can interact with transferrin receptor 1 (TFR1), decreasing its affinity for transferring and consequently modulating iron absorption in enterocytes (2). TFR2 is expressed predominantly in the liver, is implicated in the uptake of iron by hepatocytes through a receptor-mediated endocytosis mechanism, and has a high degree of homology with TFR1 (3).

Several mutations in the *HFE* gene have been associated with HH in different populations (4). The *HFE* G845A (C282Y) mutation is frequent in a healthy population from Northern Europe (10%) (1), but is rare or absent in African, Asian, South Pacific, and Aboriginal Australian populations (5). Higher frequencies of C282Y were found in individuals with HH, decreasing from Northern to Southern Europe, with the highest percentage occurring in Brittany (96%) (6) and the lowest in Italy (64%) (7) and Greece (39%) (8).

Two other *HFE* mutations (C187G and A193T) are characterized by substitutions of histidine to aspartic acid at position 63 (H63D) (9) and serine to cysteine at position 65 (S65C), respectively (10). The *HFE* 63D allele in the absence of the *HFE* 282Y allele was shown to be associated with a low risk for HH. However, the *HFE*

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H63D mutation is inherited in heterozygosis with the *HFE* C282Y mutation, and the carrier has an elevated risk of developing HH compared to an individual with the *HFE* 282YY genotype (7,11).

TFR2 gene mutations are less frequent than *HFE* mutations. The *TFR2* Y250X mutation, detected in a Sicilian family (12), is a nonsense mutation characterized by impairment of TFR2 protein and, consequently, alterations in iron regulation. The Q690P mutation was detected in a Portuguese man and in two of his family members with the HH phenotype (13). These two mutations in *TFR2* were demonstrated in HH patients, but their frequencies in healthy individuals are unknown.

A few studies have evaluated the frequency of the *HFE* C282Y mutation in Brazilian healthy individuals, but the effect of this mutation on iron status in Brazilian healthy blood donors is not known (14-16). In addition, the frequency of *TFR2* mutations in the Brazilian healthy population is also not known. The objectives of this study were to determine the frequencies of functional mutations in the *HFE* and *TFR2* genes, and to identify their relationship to iron status in blood donors.

Material and Methods

Study population

This study included 542 Brazilian healthy volunteers randomly selected among blood donors from Hemocentro da Santa Casa de São Paulo, SP, Brazil, in 2005. Two Ethics Committees (Santa Casa and Faculdade de Ciências Farmacêuticas) approved the study protocol, and written informed consent was obtained from all participants prior to entering the study.

Demographic data and the frequency of previous donations were obtained by a structured interview. Each person self-identified as White, Intermediate, Black, or Yellow according to the skin color categories defined by the Brazilian Census (17). The ethnic self-identification classification was compared with ancestry informative markers in a recent Brazilian study, which concluded that Brazilian individuals should be considered as a heterogeneous population (18,19).

Blood donors were divided into three groups according to the frequencies of blood donations. The individuals who donated blood for the first time were classified as first-time donors. Individuals who had donated blood more than once in the last 12 months were classified as frequent donors. Last, individuals who donated blood any time prior to the last 12 months were classified as sporadic blood donors.

Only individuals with hematocrit values higher than 39 and 38% for men and woman, respectively, were accepted for blood donation and enrolled in the study (20). Blood donors with altered liver function and/or with hepatitis C were excluded from the study.

Blood sampling and laboratory determinations

Peripheral venous blood was drawn using BD Vacutainer System® containing K_3EDTA (Becton Dickinson, USA) for blood cell counts and genetic analysis. An additional blood sample was collected into a BD Vacutainer System® without anticoagulant for measurements of serum iron (SI), serum ferritin (SF), total iron-binding capacity (TIBC), alanine (ALT) and aspartate (AST) aminotransferase activities, and hepatitis C and B immunological markers.

SI, TIBC, ALT, and AST were measured by colorimetric and enzymatic assays using the automated system Advia 1650® (Bayer Diagnostics, USA). Transferrin saturation (TS) was estimated as the ratio between SI and TIBC and reported as percentage. SF was determined by an immune assay using the Axsym System® (Abbott Laboratories, USA). Hepatitis C and B were detected by immune assays using the Murex anti-HCV® kit (Murex Biotech S.A., South Africa) and the Hepanostika anti-HBc Uni-Form® and Hepanostika HbsAg Uni-FormII® kits (BioMérieux, The Netherlands), respectively.

Genetic analysis

Genomic DNA was isolated from whole blood by a salting-out method previously reported (21). *HFE* C282Y and *TFR2* Y250X mutations were detected by the polymerase chain reaction and restriction fragment length polymorphism analysis (PCR-RFLP) as previously described (22,23). The primer sequences (Invitrogen, Brazil) for genotyping the *HFE* (H63D and S65C) and *TFR2* Q690P mutations were modified in the present study using the Primer Premier version 5.0 software (Sigma Chemical Co., USA) based on previously published sequences (10,13). For amplification of the *HFE* H63D and S65C mutations, we used the following primer sequences: forward 5'-TGTTGCTCTGTCTCCAGGTTCA-3' and reverse 5'-CACAACCACAGCAAGGGTATGT-3', using 34 cycles and a hybridization temperature of 63°C. The *TFR2* Q690P mutation was amplified using the following primer sequences: forward 5'-CTCCAGCACTCTGTCCTCGTCTA-3' and reverse 5'-GCGATCAAAGTGATGAAATGGA-3', using 30 cycles and a hybridization temperature of 60°C. The PCR assays were carried out using the Eppendorf Mastercycler (Eppendorf, Germany).

PCR products were digested with the *Rsa*I, *Mbo*I, *Hin*fI, and *Bfa*I endonucleases (New England Biolabs Inc., USA) to detect the *HFE* C282Y, *HFE* H63D, *HFE* S65C, and *TFR2* Y250X mutations, respectively. The restriction assay for *TFR2* Q690P genotyping was carried out by double digestion with *Bfa*I and *Hpa*II (New England Biolabs Inc.) to differentiate fragments of similar size after *Hpa*II digestion. Restriction fragments were analyzed by electrophoresis on 2% agarose gel (*HFE* C282Y, *HFE* H63D, *HFE* S65C, and *TFR2* Y250X) and 8% polyacrylamide gel (*TFR2* Q690P).

Statistical analysis

Hardy-Weinberg equilibrium was determined for all genotypes using the chi-square test. The distributions of concentrations of SI, TIBC, TS, and SF were all skewed. Log-transformation removed this skew and the variables became normally distributed in order to be used in the parametric tests. Age was skewed because of the difference between groups and was analyzed by the Mann-Whitney test.

The chi-square test or the Fisher exact test was also used to compare the frequencies of donations, genotypes for the *HFE* C282Y, H63D and S65C mutations, and ethnic groups according to blood donor gender.

One-way analysis of variance (ANOVA), adjusted by age and number of blood donations, was used to compare the mean concentrations of SI, TIBC, TS, and SF from the female and male blood donor groups, formed according to genotypes for three *HFE* mutations. The same comparisons were performed in groups formed according to the number of blood donations. When ANOVA was significant, the Tukey-Kramer post-test was performed to determine significant differences among groups.

To assess the simultaneous relationship between the various predictors of SF, TS, and TIBC in the male donors at their first blood donation (as dependent variables), three models of multiple linear regression analysis were used. The independent variables were: age, White versus non-White individuals (the non-White group was the reference, and 2 Yellow individuals were excluded from this model), CY versus CC genotype for *HFE* C282Y mutation (the CC genotype was the reference), HD + DD versus HH genotype for the *HFE* H63D mutation (the HH genotype was the reference) and SC versus SS genotype for the *HFE* S65C mutation (the SS genotype was the reference).

All statistical analyses were carried out using statistical analysis software (SAS - Statistical Analysis System for Windows, version 8.02, SAS Institute Inc., USA), with the level of significance set at $P < 0.05$.

Results

General data of the sample population

Of the 542 eligible subjects, 371 (68.5%) were male donors. The distribution of age, color groups, blood donation frequency groups, frequencies of *HFE* genotypes and *HFE* combined genotypes are presented in Table 1.

The frequencies of blood donation differed between genders. The percentage of women (35.7%) was significantly higher than the percentage of men (20.8%, $P < 0.001$) in the first-time donor group, while there was a higher percentage of men (45.0%) than women (32.7%, $P < 0.001$) in the frequent donor group (Table 1).

Frequencies of the *HFE* and *TFR2* mutations

The frequencies of the *HFE* 282Y, *HFE* 63D and *HFE* 65C alleles were 2.1, 13.6, and 0.6%, respectively. No difference was found in the allele frequencies in male and female blood donors according to the skin color ($P > 0.05$). The genotype distributions for *HFE* mutations were in Hardy-Weinberg equilibrium (Table 1). The *TFR2* 250X and *TFR2* 690P alleles were not detected in a subpopulation of donors

Table 1. Demographic and genetic characteristics of the blood donors who participated in the present study.

	Women, N (%)	Men, N (%)
Gender	171	371
Age (years)***	31.3 (29.8-32.9)	32.6 (31.6-33.7)
Skin color*		
White	91 (53.4)	201 (54.2)
Intermediate	56 (32.9)	110 (29.7)
Black	21 (12.4)	58 (15.6)
Yellow	2 (1.3)	2 (0.5)
Frequency of blood donations**		
First time	61 (35.7)	77 (20.8)
Sporadic	54 (31.6)	127 (34.2)
Frequent	56 (32.7)	167 (45.0)
HFE C282Y genotype**		
CC	164 (95.9)	355 (95.7)
CY	7 (4.1)	16 (4.3)
HFE H63D genotype*		
HH	130 (76.0)	275 (74.1)
HD	38 (22.2)	89 (24.0)
DD	3 (1.8)	7 (1.9)
HFE S65C genotype*		
SS	171 (100.0)	365 (98.4)
SC	0	6 (1.6)
HFE combined genotype C282Y/H63D/S65C*		
CC/HH/SS	124 (72.5)	256 (69.0)
CC/HD/SS	37 (21.6)	86 (23.2)
CY/HH/SS	6 (3.5)	13 (3.5)
CC/DD/SS	3 (1.8)	7 (1.9)
CC/HH/SC	0	6 (1.6)
CY/HD/SS	1 (0.6)	3 (0.8)

N = number of subjects. Age = reported as the geometric mean; 95%CI in parentheses. The genotype distribution for *HFE* gene mutation of female and male blood donors was in Hardy-Weinberg equilibrium. *Fisher exact, **chi-square, and ***Mann-Whitney tests were performed. There was a significant difference between frequencies of blood donations according to gender ($P < 0.001$). Other comparisons were non-significant ($P > 0.05$).

from this study (212 donors for the *TFR2* Y250X mutation and 516 for the *TFR2* Q690P mutation).

Relationship between *HFE* mutations and iron status

SI, TIBC, TS, and SF were not associated with the *HFE* C282Y or H63D genotype in women (P > 0.05; Table 2). The 65C allele was not detected in women.

Interestingly, TIBC was lower in men carrying the *HFE* 282CY genotype when compared with the 282CC genotype carriers (P = 0.003; Table 2). In addition, higher SI and TS values were found in men carrying at least one *HFE* 63D allele (HD plus DD genotypes) than in HH genotype carriers (P < 0.05). No relationship was found between *HFE* S65C genotype and iron status in men (Table 2).

Relationship between *HFE* mutations and iron status according to blood donation frequency

The lack of association between the *HFE* C282Y and H63D genotypes and the SI, TIBC, TS, and SF data for

Table 2. Iron status according to *HFE* C282Y, H63D, and S65C mutations in blood donors.

	Serum iron (µg/dL)	TIBC (µg/dL)	Transferrin saturation (%)	Serum ferritin (µg/L)
Women				
HFE C282Y				
CC	75.1 (69.6-81.0)	339.4 (328.9-350.3)	21.9 (22.0-23.8)	28.1 (24.0-32.9)
	N = 164	N =164	N = 164	N = 155
CY	94.3 (69.0-128.9)	333.8 (264.6-421.1)	28.2 (19.3-41.2)	24.8 (11.3-54.5)
	N = 7	N = 7	N = 7	N = 7
HFE H63D				
HH	75.5 (69.4-82.1)	339.9 (327.8-352.3)	22.0 (20.0-24.2)	27.3 (22.9-32.4)
	N = 130	N = 130	N = 130	N = 125
HD + DD	76.9 (65.9-89.6)	336.9 (216.0-359.0)	22.7 (19.4-26.5)	30.5 (21.8-42.6)
	N = 41	N = 41	N = 41	N = 37
Men				
HFE C282Y				
CC	87.8 (84.4-91.3)	311.3 (305.3-317.3)	28.0 (26.8-29.2)	90.4 (82.4-99.1)
	N = 355	N = 355	N = 355	N = 328
CY	86.2 (70.0-106.1)	262.6 (207.0-333.3)	31.1 (24.1-40.3)	133.0 (92.3-191.6)
	N = 16	N = 16	N = 16	N = 16
P		0.003		
HFE H63D				
HH	88.3 (81.4-89.3)	307.8 (300.1-315.7)	27.4 (26.0-28.8)	88.7 (80.1-98.3)
	N = 275	N = 275	N = 275	N = 256
HD + DD	95.2 (89.2-101.6)	312.4 (301.2-324.1)	30.3 (28.3-32.4)	102.3 (84.9-123.4)
	N = 96	N = 96	N = 96	N = 88
P	0.016		0.048	
HFE S65C				
SS	87.6 (84.2-91.1)	309.5 (303.0-316.1)	28.0 (26.8-29.2)	91.7 (83.7-100.4)
	N = 365	N = 365	N = 365	N = 338
SC	97.3 (73.3-129.2)	281.6 (241.0-328.9)	34.3 (25.0-46.9)	111.7 (61.2-203.9)
	N = 6	N = 6	N = 6	N = 6

Data are reported as geometric mean with 95%CI in parentheses. N = number of subjects; TIBC = total iron-binding capacity. The statistical analyses were performed on the log-transformed variables. ANOVA was adjusted by the number of donations in the last 12 months and blood donor age. The *HFE* 65C allele was not detected in female blood donors.

women was independent of first blood donation (P > 0.05; Table 3).

First-time male blood donors carrying the *HFE* 282CY genotype had lower TIBC (P < 0.001) and higher TS (P = 0.020) values than the 282CC carriers (Table 3). In this group, the *HFE* 63D allele (DD plus 63HD genotypes) was associated with increased SF concentrations (P = 0.015). On the other hand, the *HFE* S65C mutation did not affect the iron variables in this sample (Table 3).

No relationship was found between *HFE* C282Y, H63D and S65C mutations and iron variables in sporadic or frequent blood donors, independent of gender (data not shown, P > 0.05).

Predictors of serum ferritin, transferrin saturation and TIBC

Multiple linear regression analysis was used to evaluate the association between *HFE* genotype and other variables on SF (model 1), TS (model 2) and TIBC (model 3) values in first-time male blood donors (Table 4). Age (3.91%, P = 0.001) and *HFE* 63HD plus 63DD genotypes (55.84%, P = 0.021) were predictors of increased SF concentra-

Table 3. Iron status according to *HFE* C282Y, H63D, and S65C mutations in first-time blood donors.

	Serum iron (µg/dL)	TIBC (µg/dL)	Transferrin saturation (%)	Serum ferritin (µg/L)
Women				
HFE C282Y				
CC	79.2 (70.0-89.7)	336.3 (318.1-355.5)	23.2 (20.2-26.7)	33.7 (26.6-42.8)
	N = 58	N = 58	N = 58	N = 56
CY	95.2 (49.1-184.5)	368.6 (189.5-716.9)	25.7 (15.8-41.8)	18.8 (2.6-135.4)
	N = 3	N = 3	N = 3	N = 3
HFE H63D				
HH	81.6 (71.6-93.1)	339.0 (317.5-362.0)	23.7 (20.2-27.8)	31.8 (23.3-43.5)
	N = 43	N = 43	N = 43	N = 42
HD + DD	76.1 (57.9-99.9)	334.8 (300.4-373.2)	22.3 (17.0-29.2)	35.1 (26.4-46.7)
	N = 18	N = 18	N = 18	N = 17
Men				
HFE C282Y				
CC	89.7 (81.0-99.2)	301.1 (288.3-314.4)	29.7 (26.6-33.1)	122.2 (105.6-141.5)
	N = 72	N = 72	N = 72	N = 65
CY	109.4 (62.7-190.9)	189.6 (82.9-434.0)	48.8 (36.2-65.8)	138.9 (58.8-328.4)
	N = 5	N = 5	N = 5	N = 5
P		<0.001	0.020	
HFE H63D				
HH	90.9 (80.4-102.8)	289.2 (268.0-312.0)	30.9 (27.1-35.2)	112.1 (96.1-130.8)
	N = 57	N = 57	N = 57	N = 53
HD + DD	90.6 (77.0-106.4)	301.0 (277.3-326.3)	30.0 (25.0-36.1)	166.0 (120.8-228.0)
	N = 20	N = 20	N = 20	N = 17
P				0.015
HFE S65C				
SS	90.4 (81.8-100.0)	291.7 (274.5-310.1)	30.6 (27.4-34.1)	125.2 (108.4-144.6)
	N = 75	N = 75	N = 75	N = 68
SC	106.7 (5.9-1923.8)	306.5 (265.1-354.3)	34.5 (1.5-777.0)	74.3 (22.3-247.0)
	N = 2	N = 2	N = 2	N = 2

Data are reported as geometric mean with 95%CI in parentheses. N = number of subjects; TIBC = total iron-binding capacity. The statistical analyses were performed on the log-transformed variables. ANOVA was adjusted by blood donor age. The *HFE* 65C allele was not detected in female blood donors.

Table 4. Influence of *HFE* C282Y, H63D, and S65C mutations and other variables on iron status in first-time male blood donors by multiple linear regression analysis.

Dependent variables	Independent variables	Parameter	Standard error	P
Serum ferritin	Intercept	-3.13	35.62	
Model 1 (N = 71)	Age	3.91	1.17	0.001
	White men	40.31	21.35	
	HFE 282CY genotype	35.13	39.80	
	HFE 63HD plus 63DD genotypes	55.84	23.57	0.021
	HFE 65SC genotype	-48.67	62.63	
Transferrin saturation	Intercept	28.96	6.29	<0.001
Model 2 (N = 77)	Age	0.08	0.20	
	White men	3.21	3.71	
	HFE 282CY genotype	17.21	7.09	0.018
	HFE 63HD plus 63DD genotypes	-1.47	3.94	
	HFE 65SC genotype	1.60	11.14	
TIBC	Intercept	317.08	26.74	<0.001
Model 3 (N = 77)	Age	-0.48	0.85	
	White men	1.98	15.76	
	HFE 282CY genotype	-83.65	30.12	0.007
	HFE 63HD plus 63DD genotypes	3.69	16.74	
	HFE 65SC genotype	-2.03	47.35	

N = number of subjects; TIBC = total iron-binding capacity. The non-White group consisted of Intermediate and Black men, excluding Yellow individuals (N = 2). Three models of multiple linear regression analysis were done. Model 1 = the independent variables were: age; White versus non-White individuals (non-White group was the reference); *HFE* 282CY versus CC genotype (CC genotype was the reference); *HFE* 63HD plus DD versus HH genotype (HH genotype was the reference); *HFE* 65SC versus SS genotype (SS genotype was the reference). Models 2 and 3 = the independent variables were the same as in Model 1.

tions, while the *HFE* 282CY genotype was associated with increased TS (17.21%, P = 0.018) and reduced TIBC (-83.65%, P = 0.007) values.

Discussion

In the present study, the frequency of the *HFE* 282Y allele (2.1%, P > 0.05) was similar to that reported in studies conducted in healthy Brazilian individuals (1.1 to 1.4%) (14-16) and in blood donors from Colombia (1.8%) (24). However, the frequency of this allele was lower (P < 0.05) than those found in blood donors from Northern Italy (4.7%) (25) and Northern Europe (5.1 to 8.2%) (5,26,27). In addition, it is known that the *HFE* C282Y mutation is rare in non-Caucasians (5,28). The low frequency of the *HFE* 282Y allele found in the present study could be explained by the high heterogeneity of the ethnic composition of the Brazilian population, which is the result of five centuries of interethnic crosses of peoples from three continents: the European colonizers mainly represented by the Portuguese, the African slaves, and the autochthonous Amerindians (18). We did not find differences in the frequency of the 282Y allele between White and Intermediate plus Black individuals (P > 0.05). However, the ethnic classification was based on self-identified skin color categorization, which could be considered a limitation of this study.

The frequency of the *HFE* 63D allele in the present study (13.6%, P > 0.05) was similar to that found in studies with blood donors from several regions of Italy (14.4-14.9%) (25,29), in White individuals from the United States (15.0%) (30), and in two studies of healthy Brazilian blood donors (10.8 and 10.9%, P > 0.05) (14,15).

The *HFE* 65C allele frequency (0.6%, P > 0.05) in this sample was similar to that found in another Brazilian study (1.0%) (15) and in blood donors from Northern Italy (0.74%) (25) and from the Faroe Islands (1.0%) (27).

To our knowledge, this is the first investigation of *TFR2* mutations in a Brazilian population. These mutations were evaluated in Brazilian individuals because it is estimated that approximately 500,000 Portuguese arrived in the country between 1500 and 1808; Brazil also received approximately 4 million immigrants from other parts of the world (Italy, Spain, and German) (31). However, the *TFR2* 690P and *TFR2* 250X alleles (initially found in Portuguese

and Italian individuals, respectively) were not detected in our sample, suggesting that *TFR2* mutations are possibly confined to restricted geographical areas (32).

We were not able to demonstrate the effects of *HFE* mutations on iron status in women. It is possible that the high volume of blood loss during menstrual cycles or the high prevalence of nutritional deficiencies in Brazilian women could be responsible (33,34). In fact, in a previous study, we found high frequencies of iron deficiency (11.9%) and iron-deficiency anemia (6.8%) in the same female blood donors (35).

Interestingly, men presented some alterations in iron parameters. It is important to emphasize that the alterations found in this study do not reflect the presence of iron overload in blood donors carrying the *HFE* 282Y or *HFE* 63D alleles. However, they were related to slightly increased TS and reduced TIBC concentrations in *HFE* 282Y allele carriers and elevated SF concentrations in *HFE* 63D allele carriers in males who donated blood for the first time.

Multiple linear regression analysis confirmed the influence of the *HFE* 282CY genotype on the TS (directly) and on the TIBC (inversely) values in male first-time blood donors. The hypothesis accepted to explain this finding is that the variant protein (with the presence of the C282Y mutation) abolishes a disulfide bridge in the HFE protein that prevents its interaction with β_2-microglobulin and TFR1, which is free to bind transferrin. Consequently, the iron absorption in enterocytes may be inadequately modulated (4).

It is possible that the effects of these mutations could be improved by interaction with other gene mutations or could possibly have an interaction with some environmental factors, leading to iron overload in the carriers in the next two or three decades of life.

Iron parameters are influenced by age, gender, and disease, as well as by biological variation within an individual (36). In the present study, we also considered the frequency of blood donations as a covariate in the statistical analyses of iron parameters (37). *HFE* mutations influenced iron status markers, such as TIBC and TS, in first-time blood donors but these relationships were not found in sporadic or frequent blood donors. Therefore, the frequency of blood donations seems to be an important variable to be considered in gene-related studies of iron status.

The *HFE* 282Y and 65C alleles were rare in Brazilian blood donors, while the *HFE* 63D allele was frequent. *TFR2* mutations were not found in this study. *HFE* C282Y and H63D mutations were associated with alterations in iron status only in male blood donors. Additional prospective studies are required to determine the effects of environmental factors and their possible interactions with *HFE* C282Y and *HFE* H63D mutations on the iron status of healthy individuals.

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