

Rute Martins · Isabel Picanço · Aidil Fonseca  
Lídia Ferreira · Odete Rodrigues · Marília Coelho  
Teresa Seixas · Armandina Miranda · Baltazar Nunes  
Luciana Costa · Luísa Romão · Paula Faustino

## The role of *HFE* mutations on iron metabolism in beta-thalassemia carriers

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**Abstract** Hereditary hemochromatosis (HH) is an autosomal recessive disorder of iron metabolism characterized by increased iron absorption and progressive storage resulting in organ damage. *HFE* gene mutations C282Y and H63D are responsible for the majority of HH cases. A third *HFE* mutation, S65C, has been associated with the development of a mild form of hemochromatosis. The beta-thalassemia trait is characterized by mild, ineffective erythropoiesis that can induce excess iron absorption and ultimately lead to iron overload. The aim of this study was to evaluate the effect of genetic markers (*HFE* mutations C282Y, H63D, and S65C) on the iron status of beta-thalassemia carriers. A total of 101 individuals heterozygous for beta-thalassemia and 101 normal control individuals were studied. The allelic frequencies of C282Y (1.5 versus 3.5%), H63D (15.3 versus 18.3%), and S65C (1.0 versus 1.5%) did not differ significantly between beta-thalassemia carriers and normal controls. Serum iron ( $P=0.029$ ) and transferrin saturation ( $P=0.009$ ) were increased in beta-thalassemia carriers heterozygous for H63D mutation. The number of subjects carrying C282Y or S65C mutations was too low to conclude their effect on the iron status. These results suggest that the beta-thalas-

semia trait tends to be aggravated with the coinheritance of H63D mutation, even when present in heterozygosity.

**Keywords** Allelic frequency · Beta-thalassemia · *HFE* gene mutations · Iron overload · Genetic modifiers

### Introduction

Hereditary hemochromatosis (HH) is an autosomal recessive disorder of iron metabolism and represents one of the most common inherited disorders in individuals of northern European descent (Dadone et al. 1982; Edwards et al. 1998). Increased intestinal absorption leads to iron overload in several organs, resulting in various symptoms such as cirrhosis, hepatocellular carcinoma, diabetes, arthritis, and cardiomyopathy (Cartwright et al. 1979). The gene responsible for HH, the *HFE* gene, encodes an HLA class I-like protein that binds to  $\beta_2$ -microglobulin and is found on the cell surface (Feder et al. 1996). Here, HFE protein also complexes with the transferrin receptor (TfR), lowering its affinity for transferrin (Feder et al. 1998). A missense mutation that changes the 282 amino acid from cysteine to tyrosine (C282Y) has been characterized as the main mutation responsible for hemochromatosis (Feder et al. 1996). The C282Y mutation disrupts a critical disulfide bond in the  $\alpha 3$  domain of the protein, preventing the HFE molecule from interacting with  $\beta_2$ -microglobulin (Feder et al. 1997; Waheed et al. 1997). A second mutation was also described in HH patients (Feder et al. 1996), changing the aspartic acid for histidine at position 63 of the protein (H63D). Although this mutation does not affect the protein cell surface expression, its ability to reduce the HFE-transferrin receptor interaction may explain its relation with iron overload (Waheed et al. 1997). Given that the presence of these mutations does not explain all the phenotypically affected HH individuals, several polymorphisms have been found in the

R. Martins · I. Picanço · L. Romão · P. Faustino (✉)  
Centro de Genética Humana,  
Instituto Nacional de Saúde Dr Ricardo Jorge,  
Av. Padre Cruz, 1649-016 Lisbon, Portugal  
E-mail: paula.faustino@insa.min-saude.pt  
Tel.: +351-217519234  
Fax: +351-217526410

A. Fonseca · L. Ferreira · O. Rodrigues · M. Coelho  
T. Seixas · A. Miranda · L. Costa  
Centro de Biopatologia, Instituto Nacional de Saúde  
Dr Ricardo Jorge, Lisbon, Portugal

B. Nunes  
Observatório Nacional de Saúde,  
Instituto Nacional de Saúde Dr Ricardo Jorge,  
Lisbon, Portugal

*HFE* gene in patients that do not carry either of these two mutations or that have at least one chromosome without an assigned mutation (Pointon et al. 2000). Among these, a substitution in exon 2, altering the 65 amino acid serine to cysteine (S65C), has been associated with the development of a mild form of hemochromatosis (Mura et al. 1999; Asberg et al. 2000).

The beta-thalassemia trait, moderately common in the southern Portugal population (Martins et al. 1993), is characterized by mild, ineffective erythropoiesis that can induce excess iron absorption and ultimately lead to iron overload (Weatherall and Clegg 1981). Since only a minority of patients develop iron overload, the hypothesis that genetic modifiers may be related to this phenotypic variability has been set (Fargion et al. 2000; Weatherall 2001). Among these, *HFE* gene mutations seem to be the most probable cause (Fargion et al. 2000). Moreover, these mutations (C282Y and H63D) are quite frequent in European populations, and H63D mutation allelic frequency reaches its higher values in southern Europe (about 20%) where the beta-thalassemia trait is also common (Weatherall and Clegg 1981; Merryweather-Clarke et al. 2000). The aim of this study was to evaluate the effect of *HFE* gene mutations C282Y, H63D, and S65C, on the iron status of beta-thalassemia carriers. Additionally, the allelic frequencies of these mutations in the beta-thalassemia carriers were compared to a normal control group.

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## Materials and methods

### Subjects

This study was performed on 101 beta-thalassemia carriers (mean age 44.4; SD 17.8) and 101 individuals selected as controls (mean age 41.0; SD 17.2), all unrelated. Due to the geographic heterogeneity of C282Y mutation in Portugal (Cardoso et al. 2001), and being that the beta-thalassemia trait is more frequent in the southern part of the country (Martins et al. 1993), all individuals studied were of southern Portugal origin. Diagnosis of the beta-thalassemia trait was defined by low mean corpuscular volume (MCV < 80 fl), low mean corpuscular hemoglobin (MCH < 27 pg), and increased HbA2 (> 3.5%). Control individuals were characterized by normal hematological parameters. All participants gave informed consent to participate in the study.

### Hematological and iron parameters

Hematological data (MCV and MCH) were obtained using a Coulter MAXM, and HbA2 was measured by high-pressure liquid chromatography (Hb-Gold). Serum iron and transferrin levels were measured using a Hitachi 911 analyser and serum ferritin with an Immulite autoanalyzer. Transferrin saturation was calculated, as described elsewhere (Wick et al. 2000).

### Molecular analysis

Total genomic DNA was isolated from peripheral blood leukocytes by a salting-out procedure (Miller et al. 1988). The beta-thalassemia genotypes were characterized by amplification refractory mutation system (ARMS) according to the conditions described by Old et al. (1990). This screening was performed to  $\beta^0$ Cd39 C  $\rightarrow$  T,  $\beta^0$ IVS-I-1 G  $\rightarrow$  A,  $\beta^+$ IVS-I-6 T  $\rightarrow$  C and  $\beta^+$ IVS-I-110 G  $\rightarrow$  A mutations, as they are the most frequent beta-globin mutations present in southern Portugal (Faustino et al. 1999). In one individual who did not carry any of the former mutations, the beta-globin gene was sequenced using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit according to the manufacturer's instructions. *HFE* gene mutations (C282Y, H63D, and S65C) were analyzed by a PCR-restriction method, as previously described (Merryweather-Clarke et al. 1997; Mura et al. 1999).

### Statistical analysis

Fisher's exact test was used to compare the allelic frequencies of the *HFE* gene mutations and the 95% confidence intervals (CI) were calculated by the binomial exact method (Zar 1996). Measurements of iron parameters are expressed by mean and 95% CI. As serum ferritin concentrations distribution approximates a log-normal distribution, geometric means were calculated. Due to the small number of individuals in the other *HFE* genotype subgroups, comparison of the iron biochemical markers was performed only between the H63D/wild-type and the wild-type/wild-type genotypes. For this purpose, the univariate analysis of variance (ANOVA) was used, including age as a covariate to control for its effect (for serum ferritin, the ANOVA was applied to the log-transformed data). For all statistical tests, the significant level was set at 5%. All statistical analyses were performed using the SPSS version 11.0 software.

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## Results

### Beta-thalassemia genotypes

All beta-thalassemia carriers (except one) revealed heterozygosity for one of the most common beta-globin mutations described in southern Portugal: 50.5% were carriers of the  $\beta^0$ Cd39 C  $\rightarrow$  T mutation, 26.7% of the  $\beta^0$ IVS-I-1 G  $\rightarrow$  A, 5.0% of the  $\beta^+$ IVS-I-6 T  $\rightarrow$  C and 16.8% of the  $\beta^+$ IVS-I-110 G  $\rightarrow$  A. These frequencies are similar to those previously described by Faustino et al. (1999). Furthermore, one individual presented heterozygosity for  $\beta^0$ Cd6 (-A) mutation, which is a very rare mutation in the southern Portugal.

**Table 1** Allelic frequency of C282Y, H63D, and S65C mutations, and 95% CI in beta-thalassemia carriers, normal controls, and random controls studied by Cardoso et al. (2001). *n* number of chromosomes

	Beta-thalassemia carriers ( <i>n</i> =202)	Normal controls ( <i>n</i> =202)	Random controls [Cardoso et al. (2001)] ( <i>n</i> =496)
C282Y	0.0148 (0.0031–0.0428)	0.0346 (0.0140–0.0701)	0.0161 (0.0070–0.0315)
H63D	0.1535 (0.1067–0.2107)	0.1832 (0.1324–0.2435)	0.1754 (0.1429–0.2118)
S65C	0.0099 (0.0012–0.0353)	0.0148 (0.0031–0.0428)	Not performed

### HFE allelic frequencies and genotypes

The allelic frequencies obtained for *HFE* gene mutations C282Y, H63D, and S65C in beta-thalassemia carriers and normal controls are presented in Table 1. Fisher's test did not reveal any statistically significant difference between beta-thalassemia carriers and controls ( $P > 0.05$ ). Nevertheless, all mutations studied had a higher frequency in controls. These frequencies were also compared with those found in a previous study by Cardoso et al. (2001) regarding random controls of southern Portugal origin. Again, no statistically significant difference was found among these three groups.

Mutational analysis for the *HFE* gene (Table 2) showed that, in beta-thalassemia carriers, the genotype wild-type/wild-type was the most frequent, accounting for 67 individuals. Compound heterozygotes C282Y/H63D and H63D/S65C together with C282Y and S65C heterozygotes represent the less-common genotypes with no more than one or two individuals. H63D/wild-type was the most frequent genotype presenting an *HFE* mutation (*n*=29 individuals). No individual homozygous for these *HFE* gene mutations was found.

### HFE mutations and iron metabolism

Mean values of biochemical iron markers were analyzed in beta-thalassemia carriers according to their *HFE* genotype, and all individuals carrying *HFE* gene mutations were compared to the wild-type/wild-type genotype (Table 2). Serum iron and transferrin saturation presented a generalized increase in the genotypes carrying *HFE* gene mutations (with the

exception of serum iron in C282Y/H63D individual). This increase was statistically significant in the H63D/wild-type ( $P=0.029$  and  $P=0.009$  for serum iron and transferrin saturation, respectively). Regarding serum ferritin, individuals heterozygous for C282Y, H63D, and S65C presented higher values than the wild-type genotype. Although this marker was greatly increased in the C282Y/wild-type group, since only two individuals were found, statistical tests were not performed.

A similar analysis was performed in the normal control group. Although the biochemical iron markers also seem to be increased with the presence of *HFE* gene mutations, this increase was not statistically significant for the H63D/wild-type genotype ( $P > 0.05$ ), the only group that allowed statistical tests to be performed (*n*=27) (data not shown).

### Discussion

The allelic frequencies found for C282Y and H63D mutations in this study resemble the values obtained by Cardoso et al. (2001) in a southern Portugal population and are in conformity with the distribution pattern found for southern European populations (Merryweather-Clarke et al. 2000). It has been suggested that thalassemia-affected Italians had a lower *HFE* prevalence than the general Italian population (Fargion et al. 2000). However, we have not found any statistically significant difference for C282Y and H63D frequencies between beta-thalassemia carriers and controls, and our results do not support the previous hypothesis.

Geographic distribution of S65C mutation is relatively unknown, and studies regarding this mutation are

**Table 2** Iron parameters of beta-thalassemia carriers according to their *HFE* genotype. *wt* wild type, *CI* confidence interval, *P* *P*-value of the ANOVA, *NA* not applicable due to the small number of individuals

HFE genotype	Number of subjects	Serum iron ( $\mu\text{g/dl}$ )			Transferrin saturation (%)			Serum ferritin (ng/ml)		
		Mean	CI	<i>P</i>	Mean	CI	<i>P</i>	Mean	CI	<i>P</i>
wt/wt	67	103.31	95.4–111.2	–	31.55	28.9–34.1	–	73.72	50.3–108.0	–
C282Y/wt	2	135.00	NA	NA	42.95	NA	NA	778.78	NA	NA
H63D/wt	29	117.90*	105.5–130.3	0.029	37.83*	33.8–41.8	0.009	99.21	52.3–188.3	0.415
S65C/wt	1	113	–	–	54.13	–	–	370	–	–
C282Y/H63D	1	102	–	–	43.17	–	–	132	–	–
H63D/S65C	1	126	–	–	31.70	–	–	32.70	–	–
Total	101									

\* $P < 0.05$

generally performed on hemochromatosis patients. However, reports by Mura et al. (1999) and Juan et al. (2001) in French and Spanish individuals, respectively, consider frequency values of 1.9 and 3.0% for this mutation in healthy controls. More recently, in a study performed in the Terceira Island population (Azores), S65C mutation was found with an overall frequency of 2.1% in control individuals (Couto et al. 2003). Similarly, we found an allelic frequency of 1.5% for S65C in the normal control group. Considering genetic heterogeneity in southern European populations where C282Y homozygotes only explain 60–80% of HH phenotypes (Merryweather-Clarke et al. 2000), the presence of S65C, as well as other *HFE* mutations, should probably be considered when screening for genetic defects in hemochromatosis patients.

This study intended to evaluate the phenotypic expression of *HFE* genotypes regarding the variation of biochemical iron markers in a group of beta-thalassemia carriers. Some studies have been conducted in order to relate beta-thalassemia and *HFE* gene mutations, but the results and conclusions obtained seem to vary. A study performed by Rees et al. (1998) where beta-thalassemia intermedia patients were screened for H63D and C282Y mutations, found that the presence of C282Y mutation greatly increased the ferritin levels of one proband. These authors first enhanced the importance of genetic screening for hereditary hemochromatosis in other iron-loading conditions. Similarly, Arruda et al. (2000) and Piperno et al. (2000) associated these two diseases but with the purpose of analyzing the effect of the beta-thalassemia trait in hemochromatosis patients. Both agreed that the beta-thalassemia trait aggravates the clinical picture of C282Y homozygotes. However, Longo et al. (1999) results suggested that the presence of a single mutation in the *HFE* gene does not influence iron loading in thalassemia-major patients. More recently, Melis et al. (2002) found significantly higher levels of ferritin in beta-thalassemia carriers who were homozygotes for H63D mutation but found no statistical difference in heterozygous H63D individuals.

Our main purpose was to analyze the effect of several *HFE* genotypes on iron biochemical markers in beta-thalassemia carriers. The results reveal that the presence of H63D mutation aggravates the individuals' iron status, presenting higher serum iron, transferrin saturation, and serum ferritin compared with the wild-type group. Age was included as a covariate in the ANOVA to assure that the effect of H63D mutation was not being influenced by age. Unfortunately, the number of individuals carrying C282Y and S65C mutations was too small to conclude their effect on iron status. Some discrepancies, such as the lowering of some parameters, can also be explained by the small number of individuals in some *HFE* genotype groups or by the fact that both male and female individuals were included in this study. Additional studies should consider a larger sample number in order to determine the effect of these or other

rare *HFE* mutations in the development of iron overload in beta-thalassemia carriers.

Our overall results demonstrate that the beta-thalassemia trait, already related with the potential development of iron overload, tends to be aggravated with the coinheritance of H63D mutation, even when present in heterozygosity.

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