GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Comparison of oxidative stress and the frequency of polymorphisms in the HFE gene between hemoglobin S trait blood donors and sickle cell disease patients

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Genet. Mol. Res. 10 (4): 3446-3454 (2011)
Received April 20, 2011
Accepted October 17, 2011
Published December 8, 2011
DOI http://dx.doi.org/10.4238/2011.December.8.4

ABSTRACT. It is well documented that Hb S and iron affect blood cells, and trigger oxidative processes and generation of free radicals with potential for lipid peroxidation. We evaluated the frequency of polymorphisms in the *HFE* gene in Hb AS blood donors and how these polymorphisms influenced lipid peroxidation and antioxidant capacity. Blood samples were collected from 211 Hb AS blood donors, 119 Hb AA blood donors as a control group, and 28 sickle cell disease patients (Hb SS). The H63D allele was found at a frequency of 10.5% in the Hb

AS samples, and the C282Y allele frequency was 0.7%. In the control group, the frequencies of the H63D and C282Y alleles were 13.4 and 2.1%, respectively. In the sickle-cell disease patients, the H63D and the C282Y allele frequencies were 10.7 and 3.5%, respectively. The frequencies of the C282Y and H63D polymorphisms in Hb AS blood donors are similar to those reported for the Brazilian population. Serum malondialdehyde values, indicative of lipid peroxidation, were highest in sickle cell patients, independent of the polymorphisms in the *HFE* gene, with significant differences, showing the influence of Hb S allele in the levels of lipid peroxidation. However, the trolox equivalent antioxidant capacity average levels, indicative of the antioxidant capacity, were reduced with significant differences, indicating that in spite of a lipid peroxidation raise, this is not followed by the increased of the antioxidant capacity, leading to oxidative stress.

Key words: Hemoglobinopathy; Hemoglobin S; Antioxidant capacity; HFE; Hemochromatosis

INTRODUCTION

According to the World Health Organization, 270 million people possess genes that determine abnormal hemoglobins (Clegg and Weatherall, 1999; Bandeira et al., 2007). Hb S, which originated as a point mutation from the GAG (glutamic acid) codon to the GTG (Valine) codon in the sixth position of the β-globin chain, is the most prevalent hemoglobin variant in Brazil. This substitution leads to alterations in the stability and solubility of the molecule (Serjeant, 1992) and promotes polymerization in conditions with low oxygen tension (Higgs and Weatherall, 1993; Weatherall and Clegg, 2001).

The cell damage caused by free radicals derived from activated oxygen species ($O_2^{\bullet-}$, H_2O_2 and $HO^{\bullet}$) is associated with a decrease in the survival of red blood cells in various types of hemolytic anemia, including drug-induced anemia, both acquired and inherited hemolytic anemia, and anemia related to heavy metals. Sickle cell erythrocyte damage is directly intensified by the concentration of intra-erythrocyte Hb S (Beutler et al., 1995; Frenette and Atweh, 2007). Any pathology that increases radical generation, whether by an increase in oxidative stress or by an imbalance of antioxidant defenses, will increase the production of activated oxygen species, and will trigger oxidative processes with free radical generation (Naoum and Souza, 2004). A similar effect was found in cases of iron overload, as a consequence of hereditary hemochromatosis. The deficiency in iron metabolism resulting from polymorphisms on the *HFE* gene leads to iron accumulation in the tissue, which creates the potential for oxidant capacity and lipid peroxidation.

Hereditary hemochromatosis (HH) is a recessive autosomal disease characterized by iron overload in the body, with a progressive accumulation of this metal in some tissues, which may lead to structural and functional damage of organs such as the heart, liver, and pancreas, as well as occasional inflammatory and oxidative processes (Ferreira et al., 2008). The clinical manifestations generally appear between 40 and 60 years of age and occur predominately in men, because the menstrual cycle and pregnancy function has a physiological control system

in women (Ferreira et al., 2008). HH is most common among populations of Northern European ancestry. It affects one out of every 200-300 Caucasians, and is likely of Celtic origin (Cullen at al., 1998; Powell, 2002; Pietrangelo, 2006). Initially, two mutations were identified in the *HFE* gene in patients with HH: the C282Y allele (in which a tyrosine residue is substituted for a cysteine residue at position 282) and the H63D allele (which results from an exchange of histidine to aspartic acid at amino acid 63), and they correspond to 90% of HH cases (de Souza et al., 2001; Beutler, 2004; Fleming and Britton, 2006; Siah et al., 2006). The reduced number of patients despite the higher frequency of the mutation suggests incomplete penetrance of this gene (Bittencourt et al., 2002; Bonini-Domingos, 2007).

After considering the reports in the literature regarding the possible oxidant effect of hemoglobin S and iron overload as a result of hemolysis and/or the inheritance of the mutations on the *HFE* gene, the aim of this study was to evaluate the frequency of the polymorphisms C2827 and H63D in the *HFE* gene in Hb S trait blood donors, and its connection to lipid peroxidation and oxidative stress.

MATERIAL AND METHODS

In this study, peripheral blood samples were used. They were collected and put into a 5% EDTA anticoagulant solution in order to analyze the hemoglobins and the mutations in the *HFE* gene, or mixed with Heparin in order tube to verify the oxidative processes. The population in this study was comprised of 211 samples from Hemoglobin S trait blood donors. One hundred nineteen samples from Hb AA blood donors were used as a negative control, and 28 samples from sickle cell disease patients (Hb SS or Hb S/beta thalassemia) were used as a positive control. The blood donors were between 18 and 65 years old, at an average of 36.41 years. In the Hb AS group, 65.55% were male, and in the Hb AA control group, 71.56% were male. Although 70% of the participants listed their ethnicity as Caucasian, 11% as black, 5% as Asian, and 14% as mixed race, the vast majority of the participants in both the studied group and the control groups were of mixed race, and were therefore classified as such, which, according to the classification of the Brazilian population, is usually some combination of African-American, Caucasian, and indigenous ancestry (Parra et al., 2003). The sickle cell patients were between 15 and 47 years, at an average of 25.48 years old. Twenty-four patients (85.71%) listed their ethnicity as black, and four patients (14.29%) listed their ethnicity as Caucasian, despite the presence of the mutant allele β^s, which is a marker of African ancestry. Out of the 28 sickle cell patients studied, 14 (50%) were male.

The hemoglobin profiles were confirmed using: hemoglobin electrophoresis in alkaline pH cellulose acetate; acidic pH electrophoresis; and high-performance liquid chromatography (HPLC) (Bonini-Domingos et al., 2006). The DNA from all the samples was extracted using a modified form of the phenol-chloroform method (Bonini-Domingos et al., 2006). The genotypes of the hemoglobins were identified using allele-specific PCR (AS PCR). All of the samples were analyzed using the Restriction Fragment Length Polymorphisms (PCR-RFLP) technique in order to identify the mutations in the *HFE* gene (Torres et al., 2008). To determine the oxidative processes, we evaluated the antioxidant capacity based on Trolox (TEAC) (Re et al., 1999) and MDA for lipid peroxidation.

In order to compare the frequency of the polymorphisms, the chi square test (χ^2) was applied to the data, with a significance level of 95% ($P > 0.05$), as well as Yates' correction,

where N < 5. To compare the allele frequencies, the chi square test was used, and the data was then verified using the Hardy-Weinberg equilibrium. The ANOVA test was used (along with Tukey's post-hoc) to compare the average values and the standard deviation of the values obtained, and also to infer the presence of oxidative processes. Student's *t*-test was used for comparisons between groups using the Statistica 7.0 software. This project received approval from the local Ethics Committee (license #29/2004) and CONEP (The National Commission on Ethics Research), and was registered as study #11586.

RESULTS

Of the 211 Hb AS blood donors, 43 (20.38%) were identified as having at least one of the two mutations in the *HFE* gene, and of these 43 donors, 28 (65.11%) were male. Out of the 119 evaluated samples from the control group with Hb AA donors, 34 (28.57%) presented with at least one of the mutations on the *HFE* gene, and of these 34 donors, 24 (70.58%) were male. As for the positive control sickle cell patient group, of the 28 patients evaluated, 8 (28.57%) were carriers of at least one of the polymorphisms in the *HFE* gene, 50% of which were men. Figure 1 illustrates this distribution.

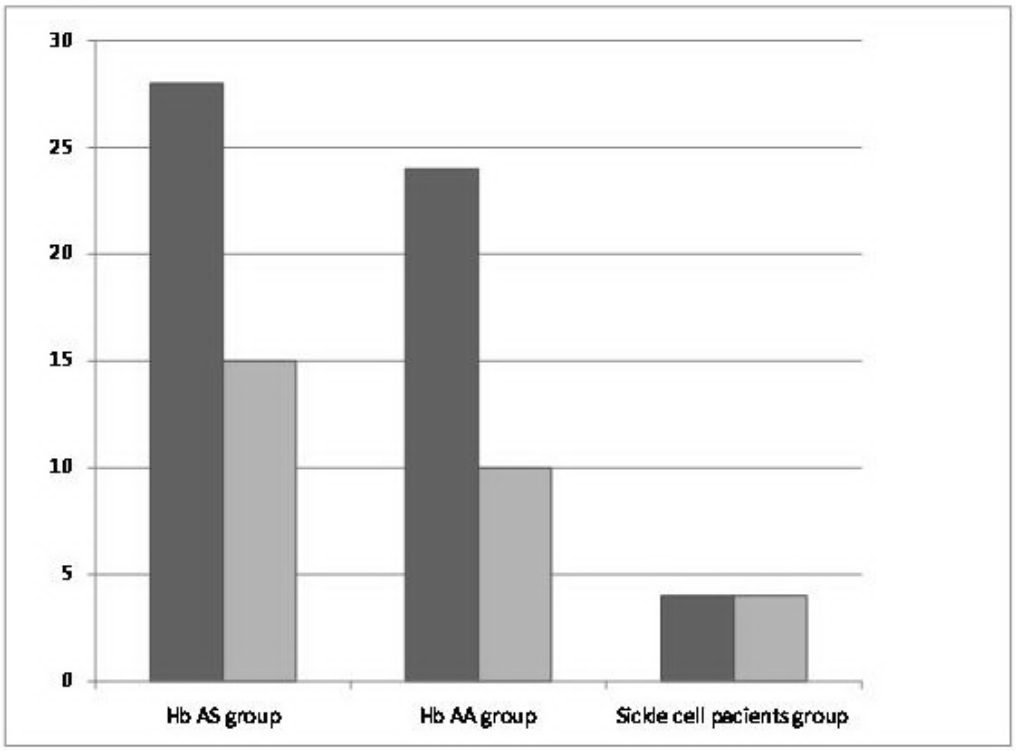

Figure 1. Separation for gender in individuals with *HFE* gene polymorphisms in the groups studied.

Among the carriers of the sickle-cell trait, 37 samples (17.54%) were heterozygous and three (1.42%) homozygous for the H63D mutation, two (0.95%) were heterozygous carriers for the C282Y mutation, and one (0.47%) was double heterozygous (C282Y/H63D). In the Hb AA group, 28 (23.53%) participants were heterozygous carriers and one (0.84%) was a homozygous carrier for the H63D mutation. Additionally, three of the Hb AA participants (2.52%) were heterozygous carriers for the C282Y mutation, and two (1.68%) were double heterozygous (C282Y/H63D). Among the sickle cell patients, six (21.43%) were identified as heterozygous for the H63D mutation, and two (7.14%) were heterozygous for the C282Y mutation. The distribution of the genotypes according to gender and hemoglobin type is shown in Table 1.

Table 1. Genotypic frequency distribution for *HFE* gene polymorphism in relation to the gender and Hb type for blood donor and sickle cell patients.

		C282Y/Wt	H63D/H63D	H63D/Wt	H63D/C282Y	Wt/Wt
Hb AA N = 119	Male	2 (1.68%)	1 (0.84%)	19 (15.97%)	1 (0.84%)	55 (46.22%)
	Female	1 (0.84%)	-	9 (7.56%)	1 (0.84%)	30 (25.21%)
Hb AS N = 211	Male	-	2 (0.94%)	26 (12.31%)	-	123 (58.28)
	Female	2 (0.94%)	1 (0.47%)	11 (5.21%)	1 (0.47%)	45 (21.32%)
Hb SS N = 28	Male	-	-	4 (14.28%)	-	10 (35.71%)
	Female	2 (7.14%)	-	2 (7.14%)	-	10 (35.71%)

WT = Wild-type allele; C282Y = mutant allele for C282Y; H63D = mutant allele for H63D.

When comparing the genotype frequencies for the mutations in the *HFE* gene, no significant differences were found between the Hb AS carriers and the control groups (χ^2 = 2.482601 and P = 0.650). We also observed that, for the three studied groups, the alleles of the mutations in the *HFE* gene are in Hardy-Weinberg equilibrium and are, therefore, representative of the population.

The allele frequency in Hb AS donors was 10.5% for the H63D allele and 0.7% for the C282Y allele. In the Hb AA control group, the frequency of H63D was 13.4%, and the frequency of C282Y was 2.1%. Among the sickle cell disease patients, H63D frequency was 10.7%, and C282Y frequency was 3.5%, as shown in Table 2.

Table 2. Allelic frequency for the C282Y and H63D *HFE* gene mutation in blood donor with Hb AS, Hb AA and sickle cell patients.

Hemoglobin	*HFE* gene mutation	N° of alleles	Mutated alleles	Allelic frequency
AS	C282Y	422	3	0.71%
N = 211	H63D	422	44	10.42%
AA	C282Y	238	5	2.10%
N = 119	H63D	238	32	13.44%
SS	C282Y	56	2	3.57%
N = 28	H63D	56	6	10.71%

When we compare the frequencies of the H63D allele in the Hb AS blood donors to each of the control groups, we found that there was no significant difference (P = 0.122) either between the Hb AS blood donors and the Hb AA carriers, or between the Hb AS blood donors

and the sickle cell patients (P = 0.890). In the comparisons involving the C282Y allele, there was again no significant difference between the Hb AS donors and the Hb AA donors (P = 0.101). Between the Hb AS donors and the sickle cell disease patients, however, we found that the allele frequency for this polymorphism was significantly higher among sickle cell disease patients (P = 0.047).

The oxidative processes were evaluated in 84 Hb AA blood donors (21 had the mutation in the *HFE* gene, and 63 did not), in 42 Hb AS blood donors (23 had the mutation in the *HFE* gene, and 19 did not), and 25 sickle cell disease patients (eight had the mutation in the *HFE* gene, and 17 did not). The difference can be contributed to the logistical complications in completing the oxidative process analysis in samples that were previously stored to avoid asking the participants for a new collection.

For MDA and TEAC values, the individuals were classified into the following six groups:

A) Individuals with Hb AA, with the mutation in the *HFE* gene; N = 21
B) Individuals with Hb AA, without the mutation in the *HFE* gene; N = 63
C) Individuals with Hb AS, with the mutation in the *HFE* gene; N = 23
D) Individuals with Hb AS, without the mutation in the *HFE* gene; N = 19
E) Individuals with Hb SS, with the mutation in the *HFE* gene; N = 8
F) Individuals with Hb SS, without the mutation in the *HFE* gene; N = 17

The MDA values were higher only among sickle cell disease patients (Table 3). In terms of MDA, we found no significant difference between individuals that were or were not heterozygous for Hb S or between those with or without mutations in the *HFE* gene, whether the mutations were alone or in coinheritance. However, the values were significantly higher in patients that were homozygous for Hb S, with or without inheritance of the mutation in the *HFE* gene.

Table 3. Medium values and standard deviation, minimum and maximum values of MDA and TEAC in the evaluated groups.

Groups	Sub-grups	MDA		TEAC	
		M ± SD (ng/mL)	Min-max (ng/mL)	M ± SD (μM)	Min-max (μM)
Hb AA	*HFE*	294.61 ± 107.52	112-578	2.07 ± 0.09	1.86-2.2
	No *HFE*	278.80 ±104.18	93-483	2.07 ± 0.11	1.78-2.31
Hb AS	*HFE*	335 ± 128.38	112-600	2.13 ± 0.03	2.04-2.21
Hb SS	No *HFE HFE*	253.63 ± 122	120-461	2.16 ± 0.05	2.07-2.27
	No *HFE*	766.87 ± 353	335-1524	2.04 ± 0.07	1.94-2.16
		903.58 ± 507	372-2362	2.05 ± 0.08	1.88-2.21

Reference values: MDA = 0-440 ng/mL; TEAC = 1.78-2.30 μM.

All TEAC values obtained were within the normal range; however, we found that there was a significant difference in presence of the allele for Hb S, whether they were with or without the mutation in the *HFE* gene, indicative of the relatively high total antioxidant capacity in heterozygous participants (Hb AS) and the relatively low in homozygous (Hb SS) when compared to the Hb AA blood donor control group. When we compare the TEAC results of Hb AS carriers, whether they had or did not have the mutation in the *HFE* gene, we also found a

significant difference, and the results were lower in cases with a coinheritance of the mutation in the *HFE* gene. Upon comparing the values obtained from the sickle cell disease patients with the HFE mutation to the sickle cell patients without it, we verified that there was no significant difference between these two groups of patients studied. Table 4 shows the results of statistic analysis for these parameters in the evaluated groups.

Table 4. ANOVA between MDA and TEAC for comparison of the studied groups.

Groups	MDA	TEAC
A x B	P = 0.5518	P = 0.9999
A x C	P = 0.2859	P = 0.0089*
A x D	P = 0.2693	P = 0.0004*
A x E	P = 0.0075*	P = 0.3565
A x F	P = 0.0001*	P = 0.4736
B x C	P = 0.0936	P = 0.0003*
B x D	P = 0.4229	P = 0.0001*
B x E	P = 0.0006*	P = 0.3129
B x F	P = 0.0001*	P = 0.4074
C x D	P = 0.0530	P = 0.0399*
C x E	P = 0.0120*	P = 0.0086*
C x F	P = 0.0003*	P = 0.0010*
D x E	P = 0.0051*	P = 0.0013*
D x F	P < 0.0001*	P < 0.0001*
E x F	P = 0.4450	P = 0.7549

P < 0.05.

DISCUSSION

The frequencies of the H63D and C282Y HFE mutations in the gene found in the Hb AS blood donors in our study agree with the findings in the reported literature for the Brazilian population (21, 24). According to Agostinho et al., 1999, C282Y is 3 to 8 times less frequent in Brazilians than in Caucasian groups of northern Europe, while the allele frequency of H63D is almost equal. This low frequency of C282Y has been found in non-Caucasian populations, or in those that possess a very mixed ethnic background. Considering that hereditary hemochromatosis predominately affects Caucasians of European descent, the maintenance of the polymorphism in the *HFE* gene may be explained by the allele fluctuation, which is attributed to the constant movement, migration, and mixing of human populations.

A study by Jeng et al. (2003), which included 89 sickle cell patients, found a genotype frequency of 2.3% for the C282Y/WT genotype, 6.8% for H63D/WT heterozygotes, and no homozygous or double heterozygous patients. According to the authors, these results agreed with those expected for the African population with which this study was completed. We found, however, that the allele frequency of the C282Y mutation was significantly higher in the sickle cell patients studied herein. We would like to highlight that, considering that this group was largely made up of individuals of African descent, and that the mutation in the *HFE* gene originated in individuals of Caucasian descent, it is likely that the admixture of the Brazilian population can explain the variation in our findings. We would like to emphasize that the manifestation of sickle cell disease varies in sickle cell patients and depends on numerous factors; however, the large majority of patients require a transfusion, which can worsen the effects of iron overload.

When we consider that blood donors represent a sample of healthy individuals in the population, and that these mutations in the *HFE* gene generally present symptoms after 40 years of age, particularly in men, the high number of male carriers of these polymorphisms should be studied and monitored early on. Today, there are more male blood donors than female blood donors, and the act of donating blood acts as a sort of protective measure, because extracting blood, usually in the form of donation, is the best form of prevention and treatment of clinical manifestations of hemochromatosis.

After considering our findings and the results reported in the literature, we believe that the presence of the allele mutation in the *HFE* gene contributes to the increase in lipid peroxidation in Hb S carriers; however, during the statistical analysis, when the influence of the S allele was eliminated, and patients with and without the mutation in the *HFE* gene were compared, the difference was not statistically significant. The results of the statistical comparisons show significantly high levels of lipid peroxidation only in patients that are homozygous for Hb S, whether the polymorphism in the *HFE* gene was present or not. These findings are evidence of oxidation potential of this mutation.

In terms of antioxidant capacity, the presence of the mutant allele in the *HFE* gene most likely contributed to the change in these parameters, because when we compared Hb AA individuals with and without the mutation in the *HFE* gene, we did not find a significant difference. After comparing Hb AS carriers with and without the mutation, however, we did find a significant difference, along with a difference in TEAC values between the two groups. Based on the data found in this study, we believe that the variation in antioxidant capacity is more connected to the presence of Hemoglobin S than it is to the presence of the polymorphisms in the *HFE* gene.

CONCLUSION

The allele frequency of the H63D mutation was higher than that of the C282Y mutation in blood donors and sickle cell patients in the region surrounding the city of São José do Rio Preto, in northeastern state of São Paulo, Brazil. The majority of the donors were of mixed race or of African ancestry.

The rate of occurrence of the H63D and C282Y mutations for the donors with Hb AA and Hb AS blood agreed with data found in the literature regarding the Brazilian population. As for the sickle cell disease patients, the frequency of C282Y found was higher, which was not expected, especially because the majority of the members of this group were of African descent although with a small frequency of C282Y.

After analyzing the values that indicate antioxidant capacity and total lipid peroxidation, we found that the presence of the allele mutation in the *HFE* gene contributes to the variation; however, its presence is not sufficient to significantly alter antioxidant capacity or lipid peroxidation. The β^s allele proved to be involved at a statistically significant rate when the patient was homozygous for the allele, and with an increase in lipid peroxidation in sickle cell disease patients. Antioxidant capacity was found to be increased in heterozygous Hb S carriers.

ACKNOWLEDGMENTS

The authors would like to thank CNPq for the financial support and Danille Deremo for English revision.

REFERENCES

Agostinho MF, Arruda VR, Basseres DS, Bordin S, et al. (1999). Mutation analysis of the HFE gene in Brazilian populations. *Blood Cells Mol. Dis.* 25: 324-327.

Bandeira FMGC, Bezerra MAC, Santos MNN and Gomes YM (2007). Importância dos programas de triagem para o gene da hemoglobina S. *Rev. Bras. Hematol. Hemoter.* 29: 179-184.

Beutler E (2004). Iron absorption in carriers of the C282Y hemochromatosis mutation. *Am. J. Clin. Nutr.* 80: 799-800.

Beutler E, Lichtman MA, Coller BS and Kipps TJ (1995). Williams Hematology. 5th edn. International Edition, New York.

Bittencourt PL, Palacios SA, Couto CA, Cancado EL, et al. (2002). Analysis of HLA-A antigens and C282Y and H63D mutations of the HFE gene in Brazilian patients with hemochromatosis. *Braz. J. Med. Biol. Res.* 35: 329-335.

Bonini-Domingos CR (2007). Aumento de ferro, hemocromatose hereditária e defeitos no gene *HFE*. O que conhecemos na população brasileira? *Rev. Bras. Hematol. Hemoter.* 29: 341-342.

Bonini-Domingos CR, Ondei LS and Zamaro PJA (2006). Hemoglobinas Similares a S no Brasil - Um Guia Prático de Identificação. 1ª ed. Editora HN, São José do Rio Preto.

Clegg JB and Weatherall DJ (1999). Thalassemia and malaria: new insights into an old problem. *Proc. Assoc. Am. Physicians* 111: 278-282.

Cullen LM, Gao X, Easteal S and Jazwinska EC (1998). The hemochromatosis 845 G→A and 187 C→G mutations: prevalence in non-Caucasian populations. *Am. J. Hum. Genet.* 62: 1403-1407.

de Souza AF, Carvalho-Filho RJ and Chebli JF (2001). Hereditary hemochromatosis. Case report and review of the literature. *Arq. Gastroenterol.* 38: 194-202.

Ferreira ACS, Oliveira VC, Caxito FA and Gomes KB (2008). Prevalence of C282Y and H63D mutations in the gene of Brazilian individuals with clinical suspicion of hereditary hemochromatosis. *Rev. Bras. Hematol. Hemoter.* 30: 379-383.

Fleming RE and Britton RS (2006). Iron Imports. VI. HFE and regulation of intestinal iron absorption. *Am. J. Physiol. Gastrointest. Liver Physiol.* 290: G590-G594.

Frenette PS and Atweh GF (2007). Sickle cell disease: old discoveries, new concepts, and future promise. *J. Clin. Invest.* 117: 850-858.

Higgs DR and Weatherall DJ (1993). Thalassaemia. In: The Haemoglobinopathies (WB Saunders, ed.). Baillière's Clinical Hematology, London, 117.

Jeng MR, Adams-Graves P, Howard TA, Whorton MR, et al. (2003). Identification of hemochromatosis gene polymorphisms in chronically transfused patients with sickle cell disease. *Am. J. Hematol.* 74: 243-248.

Naoum PC and Souza PC (2004). Avaliação dos produtos da degradação oxidativa da Hb S nos genótipos SS, SF (S/beta 0 talassemia) e AS, em comparação com hemoglobinas normais. *Rev. Bras. Patol. Med. Lab.* 40: 249-259.

Parra FC, Amado RC and Lambertucci JR (2003). Color and genomic ancestry in Brazilians. *PNAS* 100: 177-182.

Percário S (1999). Dosagem do Dialdeido Malônico. NEWSLAB, 46-50.

Pietrangelo A (2006). Hereditary hemochromatosis. *Biochim. Biophys. Acta* 1763: 700-710.

Powell LW (2002). Diagnosis of hemochromatosis. *Semin. Gastrointest. Dis.* 13: 80-88.

Re R, Pellegrini N, Proteggente A, Pannala A, et al. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med.* 26: 1231-1237.

Serjeant GR (1992). Sickle Cell Disease. 2nd edn. Oxford University Press Inc., New York.

Siah CW, Ombiga J, Adams LA, Trinder D, et al. (2006). Normal iron metabolism and the pathophysiology of iron overload disorders. *Clin. Biochem. Rev.* 27: 5-16.

Torres FR, Souza-Neiras WC, D'Almeida Couto AA, D'Almeida Couto VS, et al. (2008). Frequency of the HFE C282Y and H63D polymorphisms in Brazilian malaria patients and blood donors from the Amazon region. *Genet. Mol. Res.* 7: 60-64.

Weatherall DJ and Clegg JB (2001). Inherited haemoglobin disorders: an increasing global health problem. *Bull World Health Organ* 79: 704-712.