

# Beta-Globin Haplotypes and Alpha-Thalassemia 3.7 kb Deletion in Sickle Cell Disease Patients From the Occidental Brazilian Amazon

Janaina Santana Carneiro<sup>a</sup>, Marilda de Souza Goncalves<sup>b</sup>, Sergio Roberto Lopes Albuquerque<sup>b</sup>, Nelson Abraham Fraiji<sup>b</sup>, Jose Pereira de Moura Neto<sup>a, c, d</sup>

## Abstract

**Background:** Sickle cell disease (SCD) includes a group of inherited red blood cell disorders. SCD patients vary widely from person to person. We describe  $\beta^S$  and  $\beta^C$  haplotypes and  $\alpha$ -thalassemia 3.7 kb genotypes from SCD patients Fundacao Hospitalar de Hematologia e Hemoterapia do Amazonas, Manaus, AM.

**Methods:** Our survey included 139 HbSS and 11 HbSC patients. Molecular genotypes have been identified by PCR-RFLP and  $\alpha$ -thalassemia 3.7 kb deletion by ASO-PCR. Male/female distribution was 42.3%/57.7%.

**Results:** The average age at enrolment was 19.1 years for HbSS and 25.85 years for HbSC. Average fetal hemoglobin was 11.27% for HbSS and 7.86% for HbSC. Anemia in HbSS patients was more severe and hemolysis twice as stronger as HbSC individuals. The frequency distribution of the most common  $\beta$ -globin haplotypes among HbSS patients was 52.5% CAR/CAR, 23.7% CAR/Ben and 18% Ben/Ben. For the HbSC group, the haplotype distribution was 36.3% CAR/CI, 27.3% Benin/CI, 18.2% CAR/CII, 9.1% CAR/CIII and 9.1% Benin/CII. Of the HbSS patients, 13.7% and 2.8% were heterozygous and homozygous for the  $\alpha$ -thalassemia 3.7 kb deletion, respectively. No HbSC patients presented the deletion.

**Conclusions:** Here we present the distribution of haplotypes  $\beta^S$  and  $\beta^C$  and  $\alpha$ -thalassemia deletion 3.7 kb in a population sample with SCD from the Occidental Brazilian Amazon. Results have been analyzed in the context of hematological and biochemical profiles.

**Keywords:** Sickle cell disease;  $\alpha$ -thalassemia; Haplotypes; Amazon

Manuscript accepted for publication December 27, 2016

<sup>a</sup>Fundacao Hospitalar de Hematologia e Hemoterapia do Amazonas, Manaus, Amazonas, Brazil

<sup>b</sup>Fundacao Oswaldo Cruz - Centro de Pesquisas Goncalo Moniz, Salvador, Bahia, Brazil

<sup>c</sup>Universidade Federal do Amazonas, Faculdade de Ciencias Farmaceuticas, Manaus, Amazonas, Brazil

<sup>d</sup>Corresponding Author: Jose Pereira de Moura Neto, Universidade Federal do Amazonas, Faculdade de Ciencias Farmaceuticas, Avenida General Rodrigo Otavio Jordao Ramos, 6200 - Coroado I, Manaus, AM, 69067-005, Brazil. Email: jp-mn@hotmail.com

doi: <https://doi.org/10.14740/jh310w>

patients

## Introduction

Sickle cell disease (SCD) is a severe genetic disorder that affects populations around the world and is characterized by the presence of hemoglobin S (HbS) inside the erythrocyte. The condition is caused by a point mutation GAG>GTG on the sixth position of the  $\beta$ -globin gene, resulting in the replacement of glutamic acid by valine in the polypeptide beta chain ( $\beta^S$ ) [1]. A variation GAG>AAG in the same locus induces a change from glutamic acid to lysine that characterizes the  $\beta^C$  chain. Homozygosis for  $\beta^S$  results in a disorder known as sickle cell anemia (HbSS); individual's heterozygous  $\beta^S\beta^C$  presents hemoglobin SC (HbSC) disease [2, 3].

HbSS patients present atypical red blood cells (RBCs) with classic sickle-shape that do not circulate properly in the microcirculation, causing blood flow obstruction, hemolysis and vaso-occlusive crisis (VOC) [4]. These events are responsible for the main clinical manifestations of the disease, as well as for significant morbidity and reduction of life expectancy [5]. HbSC patients present target-shape RBC and manifest similar clinical features of those with HbSS, however, in a lower frequency and intensity [6, 7].

Hematologic and biochemical parameters, as well as clinical manifestation of both HbSS and HbSC diseases, are influenced by genetic factors such as haplotypes of the  $\beta$ -globin gene cluster and the  $\alpha$ -thalassemia 3.7 kb deletion [8-11]. Different haplotypes linked to the  $\beta^S$  globin gene have been described in SCD patients from Senegal (Sen), Benin (Ben), Central African Republic (CAR), Cameroon (Cam) and Arab-Indian (Arab) and have been named according to the geographical region or ethnic group in which they have been originally identified. Among HbSC patients, haplotype groups have been characterized as Sen/I, Sen/II, Sen/III, Ben/I, Ben/II, Ben/III, Car/I, Car/II, and Car/III [8, 9, 12].

The presence of  $\alpha$ -thalassemia in patients with SCD is associated to milder phenotypes, as the polymerization potential of HbS and HbC decreases, leading to the presence of fewer dense cells with little deforming and increased hematocrit, reducing the vaso-occlusive events and preserving spleen function [6, 9, 13].

Although molecular characterization surveys of popula-

**Table 1.** Hematologic and Biochemical Parameters of Patients With Hemoglobin SS and SC From Manaus, Amazon, Brazil

Hematological and biochemical parameters	HbSS DP	HbSC DP	P-value
HbF (%)	9.92 ± 6.39	3.42 ± 2.74	< 0.001
RBCs (× 10 <sup>6</sup> /mm <sup>3</sup> )	2.90 ± 0.69	4.13 ± 0.62	< 0.001
Hemoglobin (g/dL)	8.52 ± 1.49	11.01 ± 1.93	< 0.001
Hematocrit (%)	25.62 ± 4.58	31.27 ± 4.83	< 0.001
MCV (fL)	89.59 ± 11.46	73.94 ± 11.43	< 0.001
MCH (pg)	29.97 ± 4.30	26.60 ± 4.06	0.002
MCHC (g/dL)	33.36 ± 1.94	35.15 ± 1.55	< 0.001
Reticulocytes (%)	3.26 ± 2.69	3.02 ± 1.90	0.892
RDW (%)	19.82 ± 2.55	17.20 ± 1.97	< 0.001
Leukocyte count (× 10 <sup>9</sup> /L)	11,964.99 ± 4,318.96	8,997.77 ± 2,562.61	0.005
Platelet count (× 10 <sup>9</sup> /L)	454.56 ± 179.14	322.00 ± 126.46	0.002
Urea (mg/dL)	20.55 ± 9.05	22.92 ± 7.28	0.366
Creatinine (mg/dL)	0.61 ± 0.17	0.80 ± 0.17	< 0.001
GGT (U/L)	31.75 ± 18.83	31.22 ± 18.43	0.937
Direct bilirubin (mg/dL)	0.90 ± 0.44	0.74 ± 0.26	0.238
Indirect bilirubin (mg/dL)	2.27 ± 1.57	1.17 ± 0.84	0.030
Glucose (mg/dL)	84.47 ± 10.02	83.92 ± 7.05	0.835
Triglycerides (mg/dL)	88.30 ± 42.25	68.87 ± 23.77	0.226
HDL cholesterol (mg/dL)	41.20 ± 13.00	52.85 ± 10.44	0.038
LDH (U/L)	521.17 ± 205.79	289.55 ± 129.18	0.001
Iron (µg/dL)	97.65 ± 49.70	69.55 ± 19.99	0.098
Ferritin (ng/mL)	925.13 ± 1415.76	241.95 ± 227.56	0.098

HbF: fetal hemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: red blood cell distribution width; GGT: gamma-glutamyl transferase; LDH: lactate dehydrogenase activity.

tions affected by SCD have been carried out in several countries including Brazil, results have been often conflicting [14-19]. In addition, for some particularly interesting regions of Brazil, such as the occidental Amazon, this molecular profile is still widely unknown. Finally, few studies have compared biochemical and hematological parameters across SCD individuals of different β-globin and α-thalassemia molecular backgrounds. Here we describe the β-globin and α-thalassemia genetic profile of a population sample of individuals affected by sickle cell and hemoglobin SC disease from the Brazilian Amazon, presented and discussed in the context of a comprehensive hematological and biochemical profile [20-23].

### Materials and Methods

The studied population sample was composed by 150 SCD patients attending the Hematological Hospital of the Fundacao Hospitalar de Hematologia e Hemoterapia do Amazonas (FHMOAM) located in the city of Manaus, capital of the state of Amazonas, Brazil. Of these, 139 were homozygotes

HbSS and 11 were double heterozygous HbSC. Gender distribution was 87 (58%) females and 63 (42%) males, with age at enrolment between 4 months and 57 years old. Average age at diagnosis was 6.5 ± 11.23 years for HbSS and 10 ± 12.52 years for HbSC individuals. Hemoglobin profiles were confirmed by high-performance liquid chromatography (HPLC) (Bio-Rad, Hercules, CA, USA). All participants or guardians (in the case of children under 18 years of age) signed the written consent form and the study was approved by the Research Ethics Committee (CEP) of the Federal University of Amazonas (UFAM) under the CAAE number 37941514.4.0000.5020.

Peripheral blood samples for the hematological and biochemical analysis were obtained during a routine appointment for follow-up. The hematological analysis was performed using the automated hematology analyzer BC-5800 (Mindray, Shenzhen, China); data were obtained for the overall count of RBCs, concentration of hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDW), total and differential leukocyte count and platelet count. Fetal hemoglobin (HbF)

**Table 2.** Hematologic Parameters Between Haplotypes Linked to Globin  $\beta^S$  Gene of Patients With Sickle Cell Disease From Manaus, Amazon, Brazil

Hematological parameters	CAR/CAR (N = 73; 52.5%)	CAR/Benin (N = 33; 23.7%)	CAR/Senegal (N = 4; 2.9%)	CAR/Cameroon (N = 2; 1.4%)	Benin/Benin (N = 25; 18%)	Benin/Senegal (N = 2; 1.4%)	P-value
RBCs ( $\times 10^6/\text{mm}^3$ )	2.76 $\pm$ 0.52	2.71 $\pm$ 0.63	3.47 $\pm$ 0.75	3.12 $\pm$ 0.13	3.04 $\pm$ 0.78	2.88 $\pm$ 0.28	0.027
Hemoglobin (g/dL)	8.18 $\pm$ 1.23	8.37 $\pm$ 1.38	9.52 $\pm$ 1.43	9.20 $\pm$ 1.31	8.60 $\pm$ 0.99	8.65 $\pm$ 2.19	0.127
Hematocrit (%)	24.56 $\pm$ 3.73	25.16 $\pm$ 4.47	28.84 $\pm$ 3.65	27.26 $\pm$ 3.81	26.10 $\pm$ 4.02	29.70 $\pm$ 3.56	0.046
MCV (fL)	90.0 $\pm$ 8.88	94.0 $\pm$ 13.45	85.4 $\pm$ 15.94	87.10 $\pm$ 9.90	87.80 $\pm$ 12.56	84.4 $\pm$ 20.36	0.196
MCH (pg)	30.07 $\pm$ 3.27	31.57 $\pm$ 5.10	28.06 $\pm$ 5.38	29.43 $\pm$ 4.82	29.20 $\pm$ 4.82	28.70 $\pm$ 9.19	0.166
Reticulocytes (%)	3.37 $\pm$ 4.41	3.76 $\pm$ 7.09	2.86 $\pm$ 3.62	1.3 $\pm$ 2.90	3.52 $\pm$ 4.09	-	0.845
RDW (%)	20.12 $\pm$ 2.21	20.03 $\pm$ 2.64	20.70 $\pm$ 2.78	16.43 $\pm$ 2.10	19.83 $\pm$ 2.38	19.25 $\pm$ 1.76	0.171
Leukocytes ( $\times 10^9/\text{L}$ )	12.01 $\pm$ 3.35	13.69 $\pm$ 5.08	7.37 $\pm$ 5.97	12.65 $\pm$ 6.01	10.35 $\pm$ 3.56	10.79 $\pm$ 3.68	0.002
HbF (%)	5.92 $\pm$ 3.05	11.14 $\pm$ 3.92	19.44 $\pm$ 9.63	17.23 $\pm$ 2.87	20.97 $\pm$ 2.75	17.80 $\pm$ 1.83	< 0.001
Platelet ( $\times 10^9/\text{L}$ )	496 $\pm$ 176	455 $\pm$ 148	315 $\pm$ 256	420 $\pm$ 112	397 $\pm$ 194	470 $\pm$ 260	0.048

RBCs: red blood cell; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; RDW: red blood cell distribution width; HbF: fetal hemoglobin.

detection was performed using the alkaline resistance biochemical test [24]. Biochemical parameters were measured as implemented in the automated A25 platform (BioSystems SA, Barcelona, Spain) and included serum concentration of urea, creatinine, direct and indirect bilirubin, glucose, triglycerides, iron and ferritin, as well as gamma-glutamyl transferase (GGT) and lactate dehydrogenase activity (LDH).

Genomic DNA was obtained from peripheral blood using HiYield Genomic DNA extraction kit (BioAmerica Inc., USA). NanoDrop ND-1000 (Isogen Life Science, the Netherlands) was used to measure DNA concentration. Seven specific loci of the  $\beta$ -globin gene located in the short arm of chromosome 11 were amplified and genotyped by PCR-RFLP, as described previously [25]. In brief, the amplified fragments were digested by the following restriction enzymes: Xmn I (position 5' of the  $\gamma^G$ ), Hinc II (pseudogene  $\psi\beta$ ), Hinf I and Hpa I (of regions 5' and 3' of the  $\beta$ -gene, respectively). After digestion, DNA fragments were separated by electrophoresis in agarose gel in 1% under a constant 80 V for 45 min and visualized under ultraviolet light.

Genotyping of the  $\alpha$ -thalassemia 3.7 kb deletion was performed by ASO-PCR as described by Baysal and Huisman, using the proposed nomenclature for normal (A + C) or mutated (A + B) genotypic profiles. The PCR products were submitted to electrophoresis (Bio-Rad, EUA) in agarose gel in 1% under a constant 80 V for 45 min and visualized under ultraviolet light. Patients were distributed into three groups: normal (A + C), heterozygous (A + C) + (A + B) and homozygous (A + B) for the deletion [26].

Statistical analyzes were performed using one-way ANOVA and Kruskal-Wallis test, as implemented in SPSS, version 22.0. P-values < 0.05 were considered significant.

## Results

Table 1 summarizes and compares the hematological and bio-

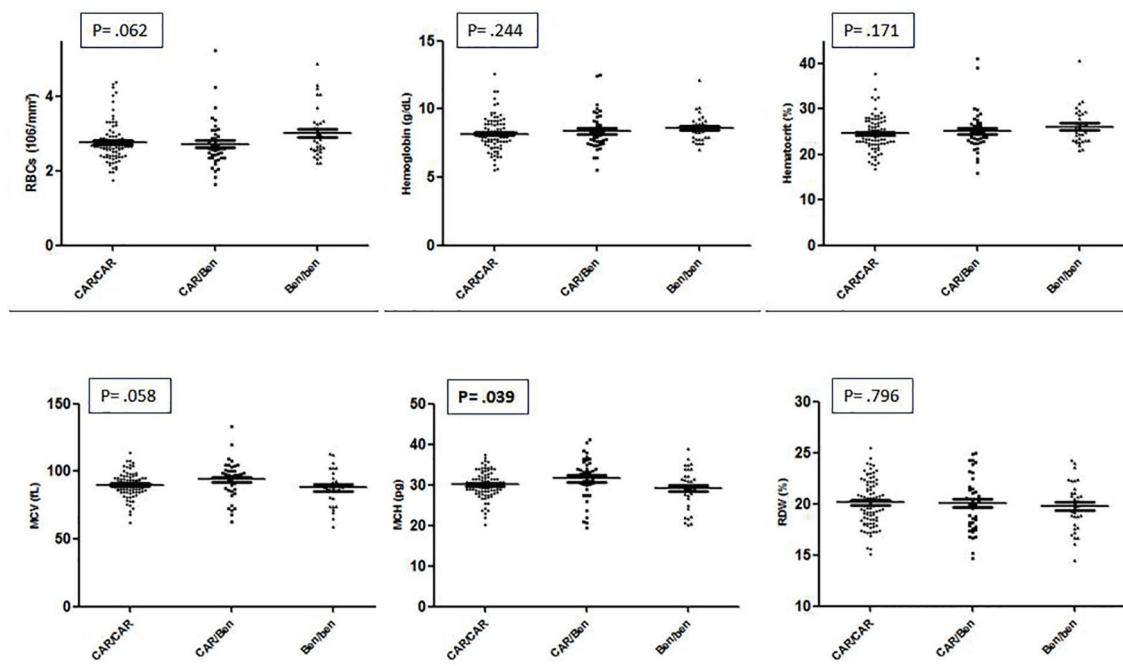
chemical parameters across the two sub-population samples of HbSS and HbSC individuals. As expected, statistically significant differences were observed for all hematological parameters except reticulocyte count. Biochemical parameters including creatinine, indirect bilirubin, lactate dehydrogenase (LDH) cholesterol and LDH activity were also different across HbSS and HbSC.

The CAR/CAR haplotype was the most frequent in HbSS individuals (73, 52.5%), followed by CAR/Benin (33, 23.7%), Benin/Benin (25, 18%), CAR/Senegal (4, 2.9%), Benin/Senegal (2, 1.4%), and CAR/Cameroon (2, 1.4%). Among the HbSC individuals, the haplotype distribution was CAR/CI (4, 36.3%), Benin/CI (3, 27.3%), CAR/CII (2, 18.2%), CAR/CIII (1, 9.1%), and Benin/CII (1, 9.1%). Due to the small number of HbSC individuals, the distribution of hematological and biochemical parameters was analyzed only for the HbSS sub-sample (Table 2). When comparing the hematological data among the most frequent haplotypes found in our study, only the MCV presented statistical significance, and the CAR/Ben haplotype presented higher concentration (Fig. 1).

A clear, statistically significant difference was observed for the HbF distribution, with heterozygous CAR/Ben and CAR/Sen presenting average levels of HbF twice and four times as higher that CAR/CAR, respectively. In addition, it is interesting to note that the leucocyte count is reduced among the four individuals CAR/Senegal. Finally, borderline significant differences were detected for RBC count, hematocrit and platelet count.

The single biochemical parameter that showed differential distribution across the haplotype groups was direct bilirubin that was significantly lower ( $P = 0.029$ ) in the CAR/Cameroon group ( $0.45 \pm 0.07$ ) as compared to CAR/CAR ( $0.88 \pm 0.35$ ), CAR/Ben ( $0.82 \pm 0.38$ ), CAR/Sen ( $0.70 \pm 0.21$ ), Ben/Ben ( $1.17 \pm 0.68$ ) and Ben/Sen ( $0.78 \pm 0.59$ ).

The  $\alpha$ -thalassemia 3.7 kb deletion was detected at a frequency of 16.5% in the HbSS group. Of these, 13.7% (19) were heterozygote's ( $-a/a$ ) and 2.8% (4) were homozygote's



**Figure 1.** Hematologic parameters between haplotypes CAR/CAR, CAR/Ben and Ben/Ben linked to globin β<sup>S</sup> gene of patients with sickle cell disease from Manaus, Amazon, Brazil.

(-α/-α). Table 3 summarizes the distribution of hematological parameters across two groups of HbSS patients presenting (carriers, both homo- and heterozygous) or not (wild type) the α-thalassemia 3.7 kb deletion. None of the patients SC presented the α-thalassemia 3.7 kb deletion. Statistically significant differences between the two groups were observed for RBC count, hemoglobin, hematocrit, MCV and MCH.

**Discussion**

The hematologic correlations between HbSS and HbSC pa-

tients included in this survey (Table 1) showed a classic profile of laboratory findings associated to sickle cell anemia and SCD. In HbSS patients, there is severe normocytic normochromic anemia associated with reticulocytosis and leukocytosis; HbSC patients presented much milder hematological changes, if any. Of note, LDH activity was above the reference interval on both groups and significantly higher in HbSS patients, suggesting intense hemolysis. Parameters such as MCV, MCH and RDW were higher in HbSS patients, corroborating surveys such as performed by Colella et al in patients with SCD attended in Hematologic and Center of Hematology and Hemotherapy (Sao Paulo, Brazil). In addition, a more pronounced

**Table 3.** Analysis of Hematologic Data in Patients of Profile SCD Carrier and Wide Type α-Thalassemia 3.7 kb Deletion From Manaus, Amazon, Brazil

Hematological parameters	Median ± DP			P-value
	Wide type (N = 116)	Carrier (N = 23)		
RBC (× 10 <sup>6</sup> /mm <sup>3</sup> )	2.77 ± 0.67	3.31 ± 0.61		0.001
Hemoglobin (g/dL)	8.37 ± 1.50	9.16 ± 1.51		0.026
Hematocrit (%)	25.18 ± 4.52	27.90 ± 4.92		0.012
MCV (fL)	90.56 ± 10.42	84.83 ± 13.99		0.012
MCH (pg)	30.61 ± 4.18	28.07 ± 4.38		0.011
MCHC (g/dL)	33.34 ± 2.15	32.99 ± 1.91		0.481
Reticulocytes (%)	3.40 ± 5.44	4.95 ± 4.70		0.387
Leukocytes (× 10 <sup>9</sup> /L)	12,098.59 ± 4,575.25	10,967.72 ± 3,435.04		0.274
Platelet (× 10 <sup>9</sup> /L)	474.66 ± 185.41	454.31 ± 201.29		0.643

RBCs: red blood cell; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration.



leukocytosis among HbSS patients confirms the participation of leukocytes in the pathophysiology of the disease, likely due to the participation in vaso-occlusive events and not necessarily in infectious processes [27].

Several haplotypes distribution analyses of variants linked to the globin  $\beta^S$  gene have been published using population samples from distinct Brazilian regions, with conflicting results. Most of these studies show a predominance of CAR as compared to Ben (the two most frequent genotypes the same result observed here [16, 20, 23, 28]. However, studies performed in population samples from northern Brazilian cities of Salvador and Fortaleza resulted in a predominance of Ben over CAR [12, 19, 21, 29, 30]. Two previous surveys have been published using population samples of  $\beta^S$  individuals resident in the Brazilian Amazonic region. The first was performed using a population sample from Belem, capital city of Para, with results somewhat distinct from the present study: three major African haplotypes (CAR, Benin and Senegal) have been identified among 30 SCD patients, with frequencies as follows: CAR/CAR 43%, CAR/Benin 47%, Ben/Ben 7% and Sen/Sen 3%. Sixty-seven percent of the  $\beta^S$  chromosomes analyzed were of the CAR type, 30% of the Benin and 3% Senegal [17]. The second focused on African descendants with SCD from three small communities from the Brazilian northern states of Para and Amapa (Curiau, Pacoval and Trombetas) and revealed an even more pronounced predominance of the CAR genotype (60%) as compared to Sen (30%) and Ben (10%) [17, 18]. These discrepancies observed across Brazilian studies involving population samples from different - and even the same - geographic region may be explained by differences in study design (mainly sample sizes) and patterns of migration of Africans to Brazil during the slavery period, and reinforce the need for local surveys.

Results of the comparative analysis of hematological and biochemical parameters across haplotype groups reveal an expected protection of the Benin and Senegal genotypes as compared to CAR, mainly due to a correlation with the levels of HbF, significantly higher in Ben/Sen haplotypes (Table 2) [9, 31, 32]. Interestingly, heterozygous CAR/Ben and CAR/Sen present average levels of HbF twice and four times as higher than CAR/CAR, respectively, suggesting a direct relationship between these variables. Importantly, this must be interpreted with caution due to the small number of CAR/Sen individuals in our sample.

Coexistence of the  $\alpha$ -thalassemia 3.7 kb deletion with SCD promotes increase of the erythrocyte's membrane/cytoplasm ratio with reduced electrolyte loss and cellular dehydration, reduced hemolysis, increase in the concentration of hemoglobin and hematocrit, decreased erythrocyte indices (MCV and MCH) and the reticulocyte count, as shown in our sample [33, 34]. The frequency of the 3.7 kb deletion of our sample of HbSS individuals does not differ from previous Brazilian studies [30, 35]. Our results confirm Rumaney et al that observed an increase in the amounts of RBCs and hemoglobin and reduced MCV in SCD patients also harboring the  $\alpha$ -thalassemia 3.7 kb deletion [36]. Pandey et al showed an increase in all parameters (erythrocytes, hemoglobin, hematocrit, MCV and MCH) in HbSS patients with  $\alpha$ -thalassemia [37].

In summary, our results show the distribution of  $\beta^S$  and  $\beta^C$  haplotypes and of  $\alpha$ -thalassemia 3.7 kb deletion in a large pop-

ulation sample of SCD patients from the Occidental Brazilian Amazon and the impact of these genotypes over hematological and biochemical parameters, contributing to the expansion of knowledge about the molecular characterization of these diseases in Brazilian populations.

## Acknowledgments

The authors wish to thank all patients with sickle cell disease attending the FHEMOAM for contributing and participating in this study. Also, the authors thank the staff of the Molecular Biology Laboratory of the Federal University of Amazonas (UFAM) and from the State University of Amazonas (UEA) for the technical support. Finally, the authors thank the Foundation for Research Support of the State of Amazonas (FAPEAM) for financial support. The sponsors of this study are public or non-profit organizations that support science in general. They had no role in gathering, analyzing or interpreting the data.

## Conflicts of Interest

The authors report no conflicts of interest.

## Sponsorships

Fundacao de Amparo a Pesquisa do Estado do Amazonas (FAPEAM) - Processo: 1094/2013-FAPEAM.

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