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Clinical and hematological parameter alterations found in sickle cell anemia heterozygotes in Brazil

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ABSTRACT. Heterozygosis for the hemoglobin S allele is a relatively common condition that is clinically benign and rarely presents clinical or hematological manifestations. Although rare, symptoms have been reported in these patients. We examined clinical manifestations and laboratory findings in HbAS individuals that could be related to the β^S haplotypes: in 31 heterozygotes, with a predominance of females and young adults, and 43 AA homozygotes considered as a control group from samples previously stored in our laboratory. We performed clinical, biochemical and hematological tests, as well as genotyping by PCR-RFLP for the identification of β^S haplotypes. Bantu and Benin haplotypes were equally frequent (n= 7, each) and 17 individuals had shown atypical haplotypes. We observed hematological alterations (e.g.

mean corpuscular volume levels) that suggest microcytic and hypochromic anemia; however, we did not find iron deficiency anemia or thalassemia. In the clinical examination, the heterozygote individuals reported pain, especially in the upper and lower limbs and joints, as the most frequent complaint. HbS heterozygotes, different from literature reports, had a significantly greater frequency of anemia-related parameters when compared to normal homozygotes.

Key words: Sickle cell anemia; HbAS; β^S Haplotypes; Microcytic anemia; Hypochromic anemia

INTRODUCTION

Sickle cell anemia has a great impact on health around the world. It is the most common hematological disease worldwide and the most prevalent in the Brazilian population, especially in the North and Northeast regions of the country. This hemoglobinopathy is characterized by alterations in the globin alleles, causing defects in hemoglobin synthesis that lead to a hemolytic anemia condition (Silva et al., 1993; Naoum, 2000; Brasil, 2002). This mutation originated 50-100 thousand years ago in Central West Africa, India and East Asia countries during the Paleolithic and Mesolithic periods (Naoum, 1997; Vogel and Motulsky, 2000).

Though all patients with sickle cell anemia present mutations in the same allele, the clinical manifestations are diverse, with different degrees of severity. Various factors have been studied to determine the causes of this diversity of manifestations. The most important are fetal hemoglobin (HbF) levels, coexistence of other hereditary hemoglobinopathies (i.e. thalassemia), and the different HbS haplotypes (Powars, 1991).

β^S haplotypes have been studied for various reasons: to determine the mutation's origin, to define mutant allele flow - useful for origin and evolution studies, and to understand the clinical evolution of patients with sickle cell anemia, since the haplotype associated with the S allele can affect clinical conditions and variations in HbF (fetal hemoglobin) levels (Nagel, 1984). The types and magnitude of complications differ among the haplotypes, suggesting a better prognosis for carriers of Senegal and Arab-Indian haplotypes, and a worse clinical evolution for carriers of Bantu and Benin haplotypes (Powars, 1991).

The frequency of the sickle cell mutation varies between 6.9 and 15.4% in individuals with African ancestry (Gonçalves et al., 2003). In Bahia state, in populations of a region called Recôncavo Baiano, heterozygotes are found at a frequency of 10.5%, while homozygotes comprised 1.24% (Silva et al., 2006). In Salvador, Bahia, the prevalence of the sickle cell mutation was estimated to be 9.8%, with 0.2% sickle cell anemia patients (ADORNO et al., 2005). According to Barboza (2013), from 2011 to 2012 in Jequié-Ba, 223 heterozygotes-AS, 101 heterozygotes-AC and two double heterozygotes (SC) were identified. The mean frequency for the sickle cell mutation was 6.45%.

One copy of HbA in heterozygotes seems to support that carriers of the sickle cell mutation are generally asymptomatic, do not show clinical conditions of sickle cell disease and that their life expectancy is similar to that of the general population. On the other hand, the sickle cell trait (heterozygotes) has been related to symptoms and abnormal conditions similar to sickle cell anemia symptoms (Tomé-Alves et al., 2000; Zago et al., 2001),

although a cause and effect (mutation-symptom) is not always apparent. So we examined whether heterozygotes show clinical symptoms similar to sickle cell disease, and investigated clinical parameters and manifestations in a population that has frequent heterozygotes.

MATERIAL AND METHODS

Sample characterization

The sample consisted of 31 individuals previously diagnosed with the sickle cell heterozygote AS from two different cities, Jequié and Jiquiriçá. The research was submitted and approved by the Institutional Research Ethics Committee - CEP/UESB (#090226/2014, CAAE 37152514.4.0000.0055). After clarifications about the research and procedures, all participants were invited to sign an Informed Consent Form or an Agreement Term.

We selected the control sample from previously collected samples stored in our laboratory. The inclusion criterion for samples was hemoglobin electrophoresis tests with a normal pattern of hemoglobin (AA), and the exclusion criterion was pregnancy, due to its association with low hemoglobin levels. The control group consisted of 43 samples within the same age group as AS heterozygotes.

The age group was between one and 79 years old; the average age was 32.54 ± 19.12 years old, and 21 participants were females. When we classified by age group, 21 individuals were from 25 to 55 years old, eight individuals were under 15 years old and two individuals were over 70 years old.

The self-declared skin color of the adults was 18 black and 13 brown, which suggests that the majority of adults have African ancestry. Data about schooling show that 10 adults did not conclude high school, and among them, three were not literate; they were the oldest participants. The information about the place of birth shows that 24 were born in the microregion of Jequié, and all 31 patients were from Bahia state.

Clinical analysis

The clinical analysis consisted of a detailed clinical examination, comprising information about general health condition and family history of disease. A demographic and socioeconomic analysis of all participants was made in order to obtain lifestyle information. For clinical tests, performed in Jequié, we had the support of a doctor from our group, and in Jiquiriçá, we had the support of doctors from the USF program (Family Health Unit), where the biological samples were collected. A questionnaire for demographic and socioeconomic data survey and the form for medical records are available (Supplementary 1).

Laboratory analysis

Determining the haplotypes of the β^S Globin

The DNA of each individual was amplified in six independent PCR reactions, each of them with a pair of primers, amplifying the sequences that contain the polymorphic sites of interest in the β^S globin allele(5'γG, γG, γA, 3'ψβ, ψβ and 5'β) (Sutton et al., 1989).

To identify haplotypes, the samples were submitted to PCR/RFLP using four restriction endonucleases BioLabs/Invitrogen (*XmnI, Hind III, HincII* and *Hinf* I). To identify haplotypes, each sample was identified by the presence (+) or absence (-) of the restriction sites in the analysis of six polymorphic sites. As a pattern for sequences, we followed what was described by Zago et al. (2000). The atypical haplotypes observed in our study were clustered in six different patterns. The analysis of origin of these haplotypes was performed by the gain or loss of one restriction site, as a result of point mutations. We compared the patterns of the atypical and typical haplotypes, observing pattern similarity (difference in one restriction site).

Biochemical and hematological analysis

The laboratory tests were outsourced to a clinical analysis laboratory where the following tests were performed: Red blood cells (Rbc), hemoglobin (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), reticulocytes (RET), uric acid (UA), urea (U), creatinine (CR), glutamic oxaloacetic transaminase (SGOT), glutamic pyruvic transaminase (SGPT), gamma-glutamyl transferase (GGT), fractions and total bilirubin (BU), fractions and total proteins (FTP), alkaline phosphatase (ALP), lactic dehydrogenase (LDH), ferritin (FER), iron (Fe), transferrin (TRANS) and hemoglobin electrophoresis.

The hematological parameters (Rbc, Hb, Ht, MCV and MCH) were categorized as normal or altered, considered the reference values for sex and age of each individual. To compare the concentrations of the different hemoglobin subtypes among individuals with sickle cell disease, we performed a new categorization, stratifying them according to the presence or absence of alterations related to anemia in the hematological parameters.

Statistical analysis

The categorized data was summarized as absolute and relative frequencies, while the continuous variables were presented as mean ± standard deviation.

Chi-square and Fisher's exact tests were performed to compare: 1) the proportions of changes in the different hematological parameters categorized between the control sample (AA individuals) and heterozygotes, as well as between individuals with different β^S haplotypes (Bantu *versus* Benin *versus* Atypical; Bantu *versus* Benin); 2) the frequency of clinical symptoms (pain, fatigue, complaint about heart, lung, liver and kidney problems, family history of sickle cell anemia) between the individuals with different β^S haplotypes (Bantu *versus* Benin *versus* Atypical; Bantu *versus* Benin). Mann-Whitney test was performed to compare the concentration of hemoglobin subtypes (HbA1, HbA2, HbS, HbF) among heterozygote individuals, to exclude the possibility of coexisting thalassemia. The variables that presented significant association were submitted to a logistic regression analysis, comparing dichotomized variables (i.e., response or outcome studied, in "control *versus* heterozygote" or "Bantu *versus* Benin"), or multinomial regression, in the case of a dependent variable with more than two levels (i.e., dependent variable with more than two strata, such as "Bantu *versus* Benin *versus* Atypical", to obtain the odds ratio (OR) with 95% confidence interval (95% CI). The regression analyses were adjusted for intervening variables when required in the Statistical Package for Social Sciences (SPSS 21.0), with $P < 0.05$.

RESULTS

We observed similar frequencies of Bantu and Benin haplotypes (seven), and 17 individuals were classified with five different variants of atypical haplotypes. When we analyzed the origin of the atypical haplotypes, we observed that all of them were related to the typical haplotypes Bantu or Benin.

The clinical results were: five individuals displayed Hb levels lower than the reference values and low Ht; eight individuals presented low MCV and seven individuals showed low MCH, suggesting a condition of microcytic and hypochromic anemia. Using the blood count data, we performed a comparative analysis between the control group (AA) and the heterozygote individuals (Table 1).

Table 1. Frequency of alterations in hematological parameters among control and HbS heterozygotes.

Hematological Parameters		Control (n = 42)	HbS Heterozygotes (n =31)
Red blood cells	NORMAL	42	31
	ALTERED	0	0
Hemoglobin	NORMAL	38	26
	ALTERED	4	5
Hematocrit	NORMAL	40	26
	ALTERED	2	5
MCV*	NORMAL	39	23
	ALTERED	3	8
MCH*	NORMAL	42	24
	ALTERED	0	7

MCV, Mean corpuscular volume; MCH, Mean corpuscular hemoglobin; (*) Significant difference in the proportions (Test SPSS; $P < 0.05$).

In the analysis of hematological parameters, we identified a significantly higher proportion of altered MCV and MCH among heterozygote individuals ($P < 0.05$; Table 1). The other parameters did not show any significant association.

Since we did not observe alterations in MCV among control individuals, which make obtaining an odds ratio (OR) unfeasible, we applied a logistic regression to the continuous data of this parameter, as well as the MCH, adjusted for age and sex. The logistic regression indicated that an increase of a unit of MCV reduces by 25% the probability of an individual presenting sickle cell disease (OR = 0.75; 95%; CI = 0.65 – 0.87; $P < 0.05$). For MCH, an increase of one unit reduces by 57% the probability of having sickle cell disease (OR = 0.75; 95%; CI = 0.29 – 0.65; $P < 0.05$).

The biochemical dosages did not show any significant association with biochemical alterations observed in sickle cell disease (homozygotes).

Table 2 presents the distribution of the hematological parameters categorized between individuals with different β^S haplotypes (Bantu *versus* Benin *versus* Atypical). We did not observe any significant association among the different haplotypes, either when comparing "Bantu *versus* Benin *versus* Atypical", or "Bantu *versus* Benin", for hematological parameters.

More than a half of the individuals (17) had a family history of sickle cell anemia (parents, children or siblings). The most important, impressive and previously unreported observation of our study, is that 22 participants related pain, mainly in the lower and upper

limbs, and joints. Furthermore, nine patients complained about fatigue and eight reported anemia conditions.

Table 2. Frequency of alterations in hematological parameters among individuals heterozygous for HbS with different haplotypes (Bantu *versus* Benin *versus* Atypical) of sickle cell disease.

Hematological Parameters		Bantu (n = 7)	Benin (n = 7)	Atypical (n = 17)
Red blood cells	NORMAL	7	7	17
	ALTERED	0	0	0
Hemoglobin	NORMAL	6	7	13
	ALTERED	1	0	4
Hematocrit	NORMAL	6	6	14
	ALTERED	1	1	3
MCV	NORMAL	5	4	14
	ALTERED	2	3	3
MCH	NORMAL	5	5	14
	ALTERED	2	2	3

MCV, Mean corpuscular volume; MCH, Mean corpuscular hemoglobin.

The distribution of the clinical symptoms and family history of sickle cell anemia among individuals with different β^S haplotypes (Bantu *versus* Benin *versus* Atypical) is shown in Table 3. We did not find significant associations for the different haplotypes, either comparing "Bantu *versus* Benin *versus* Atypical", or "Bantu *versus* Benin".

Table 3. Characterization of clinical symptoms and family history of sickle cell anemia among individuals with different β^S haplotypes (Bantu *versus* Benin *versus* Atypical).

Clinical symptoms/HAF*		Bantu (n = 7)	Benin (n = 7)	Atypical (n = 17)
Pain	NO	3	3	3
	YES	4	4	14
Fatigue	NO	6	5	11
	YES	1	2	6
Neurological problems	NO	6	7	13
	YES	1	0	4
Heart problems	NO	6	7	16
	YES	1	0	1
Lung problems	NO	5	7	16
	YES	2	0	1
Liver problems	NO	6	7	17
	YES	1	0	0
Kidney problems	NO	7	7	17
	YES	0	0	0
Anemia	NO	4	7	12
	YES	3	0	5
HAF*	NO	3	3	8
	YES	4	4	9

HAF, Family history of sickle cell anemia

DISCUSSION

The findings of our study, which analyzed the haplotypes of sickle cell anemia in heterozygotes, indicate laboratory alterations and clinical manifestations in this group.

The typical haplotypes were Bantu and Benin, both with the same frequencies (seven individuals). We also identified 17 participants with atypical haplotypes, of which 15 were probably originating from Bantu or Benin haplotypes, and only two were probably

derived from Cameroon or Bantu haplotypes; which is expected based on the geographical distribution of black people in Bahia (Verger, 1968). Nascimento (2014) investigated the β^S haplotypes in homozygotes (SS) in the same region of Jequié, and obtained similar results for atypical haplotypes. According to Zago et al. (2000), these atypical haplotypes are produced by various genetic mechanisms, recombination between two β^S typical haplotypes being the most common. The emergence of atypical haplotypes by recombination between typical haplotypes is probably due to a high degree of miscegenation (Bortolini and Salzano, 1999).

A marker of the phenotypic heterogeneity of sickle cell anemia is the influence of the β^S globin haplotypes on the clinical condition (Powars and Hiti, 1993; Silva and Gonçalves, 2010). A few studies performed with homozygotes (SS) in Brazil reported correlation between clinical manifestations of sickle cell anemia, fetal hemoglobin (HbF) and the haplotype of beta-S globin allele, including more vaso-occlusive crises and more infections in populations with the Bantu haplotype (Figueiredo et al., 1996; Adorno et al., 2004; Lyra et al., 2005; Silva Filho et al., 2012). Various reports disagree in the association between haplotypes and an increased risk of stroke; (Rodrigues et al., 2016), and Bandeira et al. (2014), attributing to the Bantu haplotype an increase in the inflammatory state due to higher concentrations of IL-6 and TNF-α cytokines, which could be an explanation for considering the Bantu haplotype to have a worse prognosis and with a greater variety of clinical manifestations.

Other studies with SS individuals highlighted the association of the Bantu haplotype with a higher incidence of clinical complications than the Benin haplotype (Nagel, 1984; Powars, 1991; Steinberg, 2001; Gonçalves et al., 2003; Adorno et al., 2008); however, our investigation did not find a significant association between the different haplotypes for clinical complaint and hematological parameters.

The hematological results of heterozygotes showed altered levels of MCV and MCH, with significantly higher frequencies than in the control group, which suggests a condition of microcytic and hypochromic anemia. This data supports the condition of eight participants, who reported anemia.

There are reports that erythrocyte morphology or erythrocyte indexes in sickle cell trait are normal (Corrons, 1994; Rodak, 1995; Mckenzie, 1996); however, others highlight that it is possible to find decreased MCV and MCH, in where differential diagnosis of the sickle cell trait must consider other causes of microcytosis, as iron deficiency anemia and thalassemia (Bain, 1997). In our research, we did not observe low levels of iron and ferritin with a suggestive condition of anemia, rejecting iron deficiency anemia as the cause of microcytosis and hypochromia. Thalassemia also was not observed in the blood exams of the heterozygotes.

Comparing hemoglobin subtypes between heterozygotes with hematological alterations, characteristic of anemia (n=8), or without these alterations (n=23), we found significantly higher concentrations of HbA1 (61.2±4.4% *versus* 58.7±1.9%; $p<0.05$) and lower levels of HbA2 (3.8±0.3% *versus* 3.3±0.4%; $p<0.05$) in individuals with a laboratory diagnosis of anemia. We did not observe significant differences for HbF concentrations (0.45±0.16% *versus* 0.56±0.43%; anemic *versus* non-anemic; $p>0.05$). According to these results, we reject the co-existence of thalassemia in heterozygotes with biochemical conditions for microcytic or hypochromic anemia.

A study performed with AS individuals to look for α-thalassemia in heterozygotes had shown altered results (microcytosis and hypochromia), which is not expected for heterozygotes. However, the authors did not consider these results relevant (Tomé-Alves et al., 2000).

Through logistic regression analysis with hematimetric parameters (MCV and MCH), we observed an inverse relationship of these parameters and the probability to develop clinical condition of sickle cell disease. As low MCV and MCH decrease, the chance is higher to have sickle cell disease.

Clinical examination showed that heterozygotes mainly experienced pain, in the upper and lower limbs, as well as in joints, as the most frequent complaint. Physical and clinical tests did not identify other pathologies. According to the literature, anemia and the recurrent pain crisis due to vaso-occlusive processes are the main clinical manifestations in sickle cell disease (Moore et al., 1996).

Although not commonly reported in sickle cell trait patients, painful episodes are reported by sickle cell disease patients when sickled red blood cells get stuck in small blood vessels, and can lead to organ damage due to oxygen deprivation in tissues and organs (Nhi, 2012). We suggest this as evidence and an explanation for pain in heterozygous individuals.

A hypothesis to explain the alterations in heterozygotes is related to the balance between the production and destruction of erythrocytes that maintain normal levels of hemoglobin in HbS individuals. This balance can be interrupted in several situations, such as hemolysis and sickling of erythrocytes (Ângulo, 2003; Motta, 2009).

Studies report that the presence of HbS allele in the genotype is enough to produce methaemoglobin through the process of deoxygenation, leading to structural alterations and alterations in plasma membrane that can cause hemolysis and anemia (Souza, 1999; Naoum, 2000). Another hypothesis to explain these alterations is genetic heterogeneity, which significantly contributes to the phenotype, and partially explains outstanding interindividual differences in severity of the disease (Nagel, 2005).

Though the current literature has not shown an association of clinical manifestations in heterozygotes for sickle cell anemia, we suggest the opposite. These results lead us to recommend that investigations continue on heterozygotes for sickle cell disease, in order to improve our knowledge about their symptoms and conditions.

CONCLUSIONS

In conclusion, our data evidence that the haplotypes Bantu and Benin present similar frequencies in the sample studied, atypical haplotypes were identified in more than a half of the sample, and they mostly originated from the haplotypes Bantu and Benin. In the biochemical findings, we did not observe significant differences when comparing the control sample with the patient sample. However, we observed significant differences in hematological parameters regarding anemia conditions in heterozygotes; logistic regression analysis demonstrated that these heterozygotes have a higher probability to develop such alterations.

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