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Research Article

Frequency and spectrum of hemoglobinopathy mutations in a Uruguayan pediatric population

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Abstract

Hemoglobinopathies are the most common recessive diseases worldwide but their prevalence in Uruguay has not been investigated. In this study, 397 unrelated outpatient children from the Pereira Rosell Hospital Center (CHPR), as well as 31 selected patients with microcytic anemia and 28 β-thalassemia carriers were analyzed for hemoglobinopathies by using biochemical and molecular biology methods. Parametric and non-parametric methods were used to compare the hematological indices between groups of genotypes. Of the 397 patients in the first group, approximately 1% (0.76% HbS and 0.25% β-thalassemia) had a mutation in the *HBB* gene and 3.3% had α-thalassemia. These mutations had a heterogeneous distribution that varied according to individual ancestry. HbS was found exclusively in individuals with declared African ancestry and had a carrier frequency of 2.2%. The frequency of α-thalassemia carriers in outpatients of European and African ancestry was 1.2% and 6.5%, respectively. In contrast, the frequency of α-thalassemia carriers in patients with microcytic anemia was 25.8%, significantly higher ($p < 0.01$) than that observed in the sample as a whole and in Afro-descendants and Euro-descendants. Significant differences were observed in the hematological parameters between individuals with thalassemia genotypes and those with a normal genotype. These results indicate that hemoglobinopathies are a relevant health problem in Uruguay.

Keywords: alpha-globin, beta-globin, hemoglobinopathies, Uruguayan population.

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Introduction

Hemoglobinopathies are the most common recessive diseases worldwide, possibly because of a selective advantage in the presence of malaria tropica (Steinberg *et al.*, 2001). Hemoglobinopathies form two main categories: those resulting from structural hemoglobin (Hb) variants and thalassemias. Among the structural Hb variants, the most frequent are HbS and HbC that are present in high frequencies in sub-Saharan Africa; HbS is also present in high frequencies in India and Saudi Arabia (Allison 1956; Steinberg *et al.*, 2001). Hemoglobinopathies resulting from these structural variants are an important health problem in the Americas, where they reflect the contribution of individuals of African descent.

Thalassemias are characterized by a decrease in or lack of synthesis of one or more globin chains resulting in a reduced rate of synthesis or absence of hemoglobin(s) (Weatherall and Clegg, 2001; Weatherall, 2004). α-Thalassemias are caused by a reduction in (a^{+}) or complete suppression of (α^{0}) α-globin chain synthesis, caused mainly by deletions of one (-α) or both (- -) *HBA* genes, although non-deletional α-thalassemia caused by small deletions or point mutations contributes to the spectrum of α-thalassemia mutations (Foglietta *et al.*, 1996; Steinberg *et al.*, 2001).

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Thalassemias occur in high frequencies in tropical and sub-tropical regions of Africa, the Mediterranean basin and Southeast Asia, where malaria is or has been endemic. However, as a result of population migration and the African slave trade these diseases have spread throughout the world and are now an important health problem in the Americas (Weatherall and Clegg, 2001). A frequent determinant of α-thalassemia is a 3.7 kb deletion (-$\alpha^{3.7}$ deletion) that affects the two *HBA* genes and results in a unique hybrid gene (HBA2-HBA1). This deletion is common in all areas with a high prevalence of thalassemia but reaches its highest frequencies in Africa and in some populations in Asia. In many cases, the hematological alterations observed in individuals with this deletion are very mild or silent.

Another frequent determinant of α-thalassemia is the 4.2 kb deletion (-$\alpha^{4.2}$ deletion) that eliminates the *HBA2* globin gene completely. This deletion is found most frequently in Asian populations but also occurs in Mediterranean regions in lower frequencies. In the Mediterranean region, the --^{MED} and -$(\alpha)^{20.5}$ deletions are the most frequent causes of α^0-thalassemias, while the deletion of a pentanucleotide (TGAGG) located at the 5' end of the IVS-I of the *HBA2* gene (denominated $\alpha^{HpHI}\alpha$) and a single point mutation at the initiation codon (ATG-ACG) of the *HBA2* gene, as well as a single point mutation (ATG-GTG) in the *HBA1* gene, are the most common non-deletional determinants of α-thalassemias in this region (Higgs *et al.*, 1989; Foglietta *et al.*, 1996; Kattamis *et al.*, 1996; Steinberg *et al.*, 2001).

β-Thalassemias, which are caused by > 190 mutations, are frequent in the Mediterranean region and southeastern Asia. Although most of these mutations are single base substitutions or small deletions or insertions within or flanking the *HBB* gene, large deletions or insertions have also be observed. Generally, in any given population, there will be a group of a few common mutations and a large number of rare ones (Huisman *et al.*, 1996; Hardison *et al.*, 1998).

The Uruguayan population originated mainly from European populations (Spanish, Italian and others), sub-Saharan African populations and native American populations but, unlike other South American countries, there are no isolated Afro-Uruguayan or native American communities. The contribution of these parental populations to the Uruguayan genetic pool is geographically heterogeneous and non-negligible, as reflected in genetic data showing contributions of 92%, 7% and 1% from European, African and Native Americans, respectively, in Montevideo, southern Uruguay; the corresponding figures for Tacuarembó, in northeastern Uruguay are 65%, 15% and 20% (Sans *et al.*, 1997). According to a Continuous Household Survey based on self-identification (I.N.E., 1997), the Uruguayan population is classified as 93.3% European, 5.9% black or mulatto, 0.4% native and 0.4% Asian. Recent data based on self-declared ancestry indicate that 87.4% of the population has exclusively European ancestry, 9.1% African ancestry, 2.9% native American ancestry and 0.6% other ancestries (Buchelli and Cabella, 2006).

In view of the ethnic origin of Uruguayan populations, it is possible that hemoglobinopathies may occur in high frequencies. An earlier study in Uruguay showed that 10% of individuals with African-ancestry were HbS/HbA carriers, a frequency similar to that observed in other countries in the Americas (Da Luz *et al.*, 2006). However, apart from the foregoing study, there are no data on the frequency and spectrum of mutations that cause hemoglobinopathies in the Uruguayan population. Moreover, there are no data on β-thalassemias although most of the Uruguayan population originated in the Mediterranean region. Approximately 19% of preschool Uruguayan children have anemia (WHO, 2008), but the causes of the disease are not always clear. The aim of this study was to contribute to our understanding of the epidemiology of hemoglobinopathies in the Uruguayan population by investigating the frequencies of structural and mutation-associated thalassemias and their impact on the prevalence of anemia in the country. This is the first study of its kind for this country.

Subjects and Methods

The sample consisted of 428 unrelated children between 2 and 15 years of age were analyzed, including a main sample of 397 outpatients randomly recruited at the Pereira Rosell Hospital Centre (CHPR) in 2006 who presented health problems unrelated to hemoglobinopathies. The children were grouped as being of either European (259) or African (138) descent based on their ancestors origin. To be classified as Afro-descendants the children had to have at least one grandparent of African origin. When their ancestry was unknown, children were classified according to their parents' self-identification, as reported in the 1996-1997 Continuous Household Survey (I.N.E., 1997). The remaining 31 blood samples belonged to children referred to the Pediatric Hematology and Oncology Service (PHOS) at the CHPR for investigation of microcytosis and hypochromia without iron deficiency and with normal levels of HbA2. The study was approved by the CHPR Ethics Committee and the children's parents gave written informed consent for them to participate in the study.

Red blood cell (RBC) indices were determined electronically with an automated cell counter (Cell Dyn 3700, USA). The presence of β-thalassemias and the most common Hb structural mutations (HbS, HbC) was investigated by electrophoresis on cellulose acetate strips at pH 8.5 (Cellogel 200μ, Cellogel Electrophoresis Co., Italy).

Genomic DNA was obtained from peripheral blood leukocytes by saline extraction (Miller *et al.*, 1988). Patients were screened for deletional and non-deletional α-

thalassemias using polymerase chain reaction (PCR)-based methods. The most common α-globin deletions ($-\alpha^{3.7}$, $-\alpha^{4.2}$, $--^{SEA}$, $--^{FIL}$, $--^{MED}$, $-(\alpha)^{20.5}$ and $--^{THAI}$) were identified by multiplex PCR while non-deletional forms were identified by restriction fragment length polymorphism (RFLP) from products amplified by PCR ($\alpha^{Hph}\alpha$, $\alpha^{NcoI}\alpha$ and $\alpha\alpha^{NcoI}$) (Hall *et al.*, 1993; Tan *et al.*, 2001). The *HBB* gene was amplified and sequenced to confirm and identify β-globin mutations (Miranda *et al.*, 1997). In samples with the HbS mutation, five polymorphic sites in the β-globin gene cluster were analyzed: 1) *HincII* 5'ε, 2) *HindIII* $^{G}\gamma$, *3) HindIII* $^{A}\gamma$, *4) HincII 5'*ψβ and 5) *HincII* 3' ψβ. Fragments containing each of these sites were amplified by PCR using previously described primers and conditions (Sutton *et al.*, 1989; Guerreiro *et al.*, 1992). Haplotypes were constructed by assuming that the presence of two common haplotypes was more probable than the combination of one common and one rare haplotype or of two rare haplotypes (Kulozik *et al.*, 1986; Long *et al.*, 1990; Castro de Guerra *et al.*, 1997; Vívenes De Lugo *et al.*, 2003). The genotypic and allelic frequencies were estimated by gene counting. The former were compared between the groups using Fisher's exact test with the Arlequin software package v.2.000 (Schneider *et al.*, 2000).

Hemoglobin values, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and RBC values were corrected for age and sex. The distribution of these values and the red blood cell distribution width (RDW) were compared among individuals with different genotypes in the main sample and among 28 carrier children previously diagnosed with β-thalassemia at the CHPR. Hemoblogin and RBC were compared by multivariate analysis of variance (MANOVA) followed by the Student-Newman-Keuls (SNK) test for pairwise multiple comparisons between groups. The Mann-Whitney test was used to compare MCV, MCH and RDW to account for non-normality. The tests were done using SPSS v.12.0 with $p < 0.05$ as the level of significance.

Results

We identified three HbS carriers (HbS/HbA) (0.75%) in the main, randomly recruited CHPR sample, all of whom had been classified as Afro-descendants. The frequency of the β^S mutation was 0.38% but increased to 1.1% for individuals of African origin (Table 1). In two individuals the HbS mutation was probably associated with the Bantu haplotype whereas the other was associated with the Benin haplotype.

Although only one sample (an individual of European ancestry) had increased HbA2, after sequencing the *HBB* gene in 15 samples with an MCV of < 75 fL and an RBC value > 5 million/μL, the only β-thalassemia mutation found (the b^0 codon 39 mutation C > T) was identified in this individual. Results for α-thalassemias in the random sample are summarized in Table 2. The only α-thalassemia mutation observed in the sample was the $-\alpha^{3.7}$ mutation, which accounted for 3.3% of the mutations and was present in a heterozygous state ($-\alpha^{3.7}/\alpha\alpha$: 3%) in 12 individuals and in a homozygous state ($-\alpha^{3.7}/-\alpha^{3.7}$: 0.3%) in one. Of the 259 Euro-descendants, three (1.2%) were heterozygous ($-\alpha^{3.7}/\alpha\alpha$); ten of the 138 Afro-descendants (7.2%) also had this mutation – nine (6.5%) were heterozygous ($-\alpha^{3.7}/\alpha\alpha$) and one (0.7%) was homozygous ($-\alpha^{3.7}/-\alpha^{3.7}$). Mycrocytosis was detected in 27 Afro-descendants and 28 Euro-descendants; nine (33.3%) of the former and only one (3.6%) of the latter had α-thalassemia. The genotypic frequencies differed significantly between Afro- and Euro-descendants ($p < 0.001$), between individuals with or without microcytosis ($p < 0.001$) and between Afro- and Euro-descendants with microcytosis ($p = 0.0047$).

Of the 31 PHOS pediatric patients who presented with microcytosis and hypochromia, 10 (32.3%) had α-thalassemia: eight (25.8%) were heterozygous for the $-\alpha^{3.7}$ deletion, one (3.2%) was homozygous for the same deletion ($-\alpha^{3.7}/-\alpha^{3.7}$) and one (3.2%) was heterozygous ($\alpha\alpha/-\alpha^{20.5}$), this being the first reported case of the $-\alpha^{20.5}$ deletion in Uruguay. These frequencies did not differ significantly from those observed in individuals with microcytosis in the random sample.

The RBC indices in individuals with α-thalassemia genotypes, β-thalassemia trait and normal α- and β-globin genotypes are shown in Table 3. MANOVA revealed significant differences in the Hb values and RBC indices between the genotypes (Pillai's trace for comparison among

Table 1 - Frequency of HbS in the main sample and in Afro-descendants.

		Genotypic frequencies			Allelic frequencies	
	N	β^A/non β^S	β^A/β^S	β^S/β^S	β^A	β^S
All[1]	397	394 (99.24)	3 (0.76)	0	0.9960	0.0038
Afro-descendants[1]	138	135(97.78)	3(2.22)	0	0.9890	0.0110
Afro-descendants[2]	40	36 (90)	4 (10)	0	0.9500	0.0500
p*		0.0458 ± 0.0018				

[1]Present study, [2]Da Luz *et al.* (2006). Percentage in parentheses. *Difference between the two groups of Afro-descendants.

Table 2 - Frequency of α-thalassemia in the main sample and in individuals grouped according to ancestry and the presence or absence of microcytosis.

	Genotypic frequencies				N	Allelic frequencies	
Subjects	αα/αα	$-\alpha^{3.7}/\alpha$	$-\alpha^{3.7}/-\alpha^{3.7}$	$-\alpha^{20.5}/\alpha\alpha$		$-\alpha^{3.7}$	αα
All	384(96.7)	12(3.0)	1(0.3)		397	0.018	0.982
Afro-descendants	128(92.8)	9(6.5)	1(0.7)		138	0.040	0.960
Euro-descendants	256(98.8)	3(1.2)			259	0.006	0.994
p		< 0.001				< 0.01	
Microcytic	45(81.8)	9(16.4)	1(1.8)		55	0.100	0.900
Non-microcytic	339(99.1)	3(0.9)			342	0.004	0.996
p		< 0.001				< 0.001	
Afro- and microcytic	18(66.7)	8(29.6)	1(3.7)		27	0.815	0.185
Euro- and microcytic	27(96.4)	1(3.6)			28	0.982	0.018
p		< 0.01				< 0.01	
Selected patients	21(67.8)	8(25.8)	1(3.22)	1(3.22)	31	0.823	0.161
p*		< 0.01				< 0.01	

Percentages in parentheses. *: Compared with Afro- and Euro-descendants. N: Number of individuals.

Table 3 - Red blood cell indices in normal patients, in patients with α-thalassemia genotypes and in β-thalassemia carriers.

Blood index	Subjects				p
	$-\alpha^{3.7}/\alpha\alpha$	$-\alpha^{3.7}/-\alpha^{3.7}$	β-thalassemia	αα/αα	
Hb (g/dL)*	$12.5 \pm 1.79^{a, b}$	12.1 ± 0.43^{a}	11.4 ± 0.88^{a}	13.7 ± 1.31^{b}	< 0.05
RBC ($x10^{12}$/L)*	5.16 ± 0.40^{a}	$5.69 \pm 0.21^{a, b}$	5.64 ± 0.50^{b}	5.01 ± 0.49^{a}	< 0.05
MCV (fL)*	74.7 ± 6.29^{a}	$63.7 \pm 0.17^{a, b}$	64.6 ± 4.07^{b}	83.1 ± 4.86^{c}	< 0.05
MCH (pg)*	23.8 ± 2.94^{a}	$20.7 \pm 0.21^{a, b}$	19.7 ± 1.05^{b}	27.5 ± 1.67^{c}	< 0.05
RDW (%)	$15.7 \pm 2.43^{a, b}$	$17.1 \pm 0.49^{b, c}$	18.2 ± 1.21^{c}	14.9 ± 1.70^{a}	< 0.05

Values are means ± SD. Hb: hemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; RBC: red blood cells; RDW: red cell distribution width. Values identified by the same letters do not differ significantly. Hb and RBC were compared with the SNK test, and MCV, MCH and RDW by the Mann-Whitney test to account for non-normality. *Analyses done using values corrected for age and sex.

genotypes: F:55.02; $p < 0.001$). Univariate analysis of Hb revealed significant differences in Hb values between the group with normal genotypes and the other groups of genotypes and between the group with the $-\alpha^{3.7}$ deletion and the other groups of genotypes; there were also significant differences in the RBC values between individuals with β-thalassemia trait and the other groups, except for $-\alpha^{3.7}/-\alpha^{3.7}$ individuals. The Mann-Whitney test revealed significant differences for the MCV and MCH indices between individuals with normal genotypes and all the other groups, and between individuals with β-thalassemia trait and the $-\alpha^{3.7}/\alpha\alpha$ genotype.

Discussion

With the exception of a previous investigation based on a small sample from the Afro-Uruguayan population (Da Luz *et al.*, 2006), this is the first study to examine the frequency of hemoglobinopathies in Uruguay, despite the fact that the population of this country originated from populations in which hemoglobinopathies are an important health problem.

Although the allelic frequency observed for the β^S mutation (0.38%) cannot be extrapolated to the Uruguayan population as a whole because the samples consisted mostly of individuals from the city of Montevideo who attended CHPR (a public hospital), it was nevertheless similar to that estimated for the Uruguayan population (0.3%) using the carrier and allelic frequencies previously reported for HbS (10% and 5%, respectively) in Afro-Uruguayans (Da Luz *et al.*, 2006). The overall frequency of HbS carriers in the sample was slightly lower than that observed in southern Brazil, a region that shares borders with Uruguay and where a carrier frequency of 1.2% has been reported for newborns (Daudt *et al.*, 2002). In contrast, there was a greater difference between the frequency in the Uruguayan population in the present study and that in populations from northeastern Brazil, where there is a greater contribution from African populations and the frequency of HbS carriers

ranges from 5.1% to 11.4% (Azevedo *et al.*, 1980; Bandeira *et al.*, 1999; Adorno *et al.*, 2005).

The frequency of the β^S mutation in the sample of African origin in the present study (1.1%) was lower than previously observed (5%) (Da Luz *et al.*, 2006); Table 1 shows that the genotypic frequencies differed significantly between the two studies. This difference can be explained by the greater African contribution in the latter study in which the population came mainly from Afro-Uruguayan social organizations and was selected based on ancestry and phenotypic traits, whereas in the present study the existence of one Afro-descendant grandparent was sufficient for an individual to be classified as Afro-Uruguayan.

The presence of Bantu and Benin haplotypes (frequencies not given here because of the small number of cases) also agreed with data from the previous study in Uruguay that showed a predominance of the Bantu haplotype. These findings are compatible with historical and demographic data on the origin of African slaves that indicate a major contribution by individuals from Bantu-speaking groups (Isola, 1975). The predominance of the Bantu haplotype has also been observed in many other countries and regions of South America (Muniz *et al.*, 1995; Pante de Sousa, 1999; Moreno *et al.*, 2002). In the Americas, the Benin haplotype is most frequent in the north of South America, the Caribbean region and North America (Antonarakis *et al.*, 1984; Arends *et al.*, 2000; Vivenes de Lugo *et al.*, 2003; Galiza Neto *et al.*, 2004; Adorno *et al.*, 2005) whereas the Senegal haplotype is found in high frequencies in isolated Amazonian Afro-Brazilian communities (Pante de Sousa *et al.*, 1999).

Interestingly, only one carrier of β-thalassemia was detected. This child had the β^0 codon 39 (C > T) mutation, the most frequent β-thalassemia mutation found in neighboring Brazil and Argentina (Martins *et al.*, 1993; Roldan *et al.*, 1997; Rossetti *et al.*, 2004). As expected, because this individual belonged to the sample without African ancestry, the frequency of β-thalassemia alleles in this group (0.41%) was similar to the global frequency observed in Spain (0.41%) and in some regions of northern Italy (0.5%), a part of the country where the lowest frequencies of β-thalassemia are found (Villegas *et al.*, 1992, 2001; Weatherall and Clegg, 2001; Calvo-Villas *et al.*, 2006).

The $\alpha^{-3.7}$ thalassemia carrier frequency (3%) in the total CHPR sample was similar to that found in Spaniards (4.4%), Portuguese (3.5%) and Italians (4.1%) (Fei *et al.*, 1989; Villegas *et al.*, 1992; Peres *et al.*, 1995; Weatherall and Clegg, 2001). When we analyzed the sub-sample with African ancestry, the $\alpha^{-3.7}$ mutation carrier frequency increased and was 6.5% higher than that for the whole sample but, as expected from the admixture, was lower than that observed in parental African populations, where the frequency of α-thalassemia carriers ranges from 11% to 50% (Weatherall and Clegg, 2001). The $\alpha^{-3.7}$ mutation carrier frequency in the sub-sample with African ancestry was also lower than that observed in Afro-Brazilian populations, where the carrier frequency ranges from 7% to 21% (Sonati *et al.*, 2001; Adorno *et al.*, 2005; Souza *et al.*, 2009; Wagner *et al.*, 2010), possibly because of a higher non-African admixture in this Afro-Uruguayan population. The α-thalassemia frequencies for Afro-descendants with microcytosis (33.3%) and the group of children with microcytosis and hypochromia (32.3%) were both similar to the frequency observed in a similar sample from the southern Brazilian state of Rio Grande do Sul (31.7%) (Wagner *et al.*, 2010) and lower than that observed for a population in southeastern Brazil in which α-thalassemia explained about 50% of the cases of microcytosis (Borges *et al.*, 2001).

The sample of 31 patients admitted to the PHOS exhibited the $-(\alpha)^{20.5}$ deletion, a mutation related to European or Asian ancestry (Huisman *et al.*, 1996; Hardison *et al.*, 1998). The presence of this deletion in a homozygous state or associated with other α-thalassemia mutations produces hydrops fetalis or HbH disease, a severe hemoglobinopathy not described to date in Uruguay.

In conclusion, the data presented here are the first on the frequency of hemoglobinopathies in a relatively large Uruguayan sample and show that these diseases have non-negligible frequencies. Although the sample analyzed was not fully representative of the Uruguayan population, about a sixth of the births in this country occur in the CHPR. The frequencies of hemoglobinopathies in this study underline the need for strategies to prevent further health complications. In light of this, Afro-Uruguayan associations have been pressing for hemoglobinopathies to be included in the universal screening of neonates (see Queiruga *et al.*, 2010, for screening data). Furthermore, data on RBC parameters in individuals with normal and thalassemia genotypes can help to detect α-thalassemia in patients suspected of having the disease. Our findings encourage us to investigate the distribution of hemoglobinopathies in a more comprehensive sample with greater African and/or European genetic contributions and to evaluate the benefits of including tests to detect hemoglobinopathies in the universal screening of neonates in Uruguay.

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