HEMATOLOGY, TRANSFUSION AND CELL THERAPY

www.htct.com.br

Original article

Clinical, laboratory, and molecular characteristics of a cohort of children with hemoglobinopathy S/beta-thalassemia

Érica Louback Oliveira [a], *André Rolim Belisário* [b], *Natiely Pereira Silva* [a], *Paulo Val Rezende* [c], *Maristela Braga Muniz* [c], *Larissa Maira Moura Oliveira* [a], *Cibele Velloso-Rodrigues* [d], *Marcos Borato Viana* [a,*]

[a] *Faculdade de Medicina/Núcleo de Ações e Pesquisa em Apoio Diagnóstico (NUPAD), UFMG, Belo Horizonte, MG, Brazil*
[b] *Centro de Tecidos Biológicos de Minas Gerais, Fundação Hemominas, Lagoa Santa, MG, Brazil*
[c] *Ambulatório do Hemocentro de Belo Horizonte, Fundação Hemominas, Belo Horizonte, MG, Brazil*
[d] *Departamento de Ciências Básicas da Vida, Instituto de Ciências da Vida, Universidade Federal de Juiz de Fora, Governador Valadares, MG, Brazil*

ARTICLE INFO

Article history:
Received 25 September 2023
Accepted 25 November 2023
Available online 19 December 2023

Keywords:
Sickle cell disease
Hemoglobin Sβ-thalassemia
Hemoglobin Sβ^+-thalassemia
Hemoglobin Sβ^0-thalassemia
Alpha-thalassemia

ABSTRACT

Introduction: Hemoglobinopathy Sβ-thalassemia (HbSβ-thal) has a wide range of clinical and laboratory severity. There is limited information on the natural history of HbSβ-thal and its modulating factors. We described the molecular, hematological, and clinical characteristics of a cohort of children with HbSβ-thal and estimated its incidence in Minas Gerais, Brazil.
Methods: Laboratory and clinical data were retrieved from medical records. Molecular analysis was performed by *HBB* gene sequencing, PCR-RFLP, gap-PCR, and MLPA.
Results: Eighty-nine children were included in the study. Fourteen alleles of β-thal mutations were identified. The incidence of HbSβ-thal in the state was 1 per 22,250 newborns. The most common β^S-haplotypes were CAR and Benin. The most frequent β^{thal}-haplotypes were V, II, and I. Coexistence of 3.7 kb *HBA1/HBA2* deletion was present in 21.3 % of children. β-thalassemia mutations were associated with several clinical and laboratory features. In general, the incidence of clinical events per 100 patient-years was similar for children with HbSβ^0-thal, IVS-I-5 G>A, and IVS-I-110 G>A. Children with HbSβ^+-intermediate phenotypes had a more severe laboratory and clinical profile when compared with those with HbSβ^+-mild ones. β^S-haplotypes and α-thalassemia did not meaningfully influence the phenotype of children with HbSβ-thal.
Conclusion: The early identification of β-thalassemia alleles may help the clinical management of these children.

* Corresponding author at: Faculdade de Medicina, Departamento de Pediatria, Universidade Federal de Minas Gerais, Avenida Alfredo Balena, 190/267, CEP 30130-100, Belo Horizonte, MG, Brazil.
E-mail address: vianamb@gmail.com (M.B. Viana).

https://doi.org/10.1016/j.htct.2023.11.002

Introduction

Sickle cell disease (SCD) is a hereditary disorder that significantly affects the world population. In Brazil, it is considered a public health issue. Although primarily determined by a single mutation in the beta globin gene [HBB:c.20A>T (p.Glu6Val)], SCD has a wide phenotypic variation attributed to genetic, environmental modulators and interactions with other variant hemoglobins (Hb).[1] The most frequent subtypes of SCD in Brazil are Hb SS, Hb SC, and Hb Sβ-thalassemia (HbSβ-thal).[2]

HbSβ-thal is a subtype of SCD characterized by clinical and laboratory heterogeneity, mainly due to different β-thal mutations and, consequently, null or different levels of residual production of HbA. HbSβ-thal is classified according to the gene expression of the β-thal allele into HbSβ^+-thal (residual HbA) or HbSβ^0-thal (absence of HbA).[3-5] In general, HbSβ^+-thal is considered to be a milder or intermediate phenotypical subtype of SCD while HbSβ^0-thal is similar to sickle cell anemia (SCA).

Although the β-thal mutation can partly explain the phenotypic variation, there is still a wide variability in the clinical severity of HbSβ-thal.[3,6] Co-inherence of alpha-thalassemia (α-thal) and beta-globin gene cluster haplotypes may influence the severity of this subtype of SCD. Haplotypes are well studied in many reports, mainly in SS patients.[7] In previous papers, we demonstrated that co-inheritance of α-thal significantly decreased the risk of cerebrovascular disease in SS children and of acute splenic sequestration in SC children.[8,9] However, the association of haplotypes or α-thal with the clinical course of HbSβ-thal has been only occasionaly reported.[10]

It is estimated that there are 60,000 people with SCD in Brazil, with a wide incidence variation between regions and states.[2] However, the incidence of HbSβ-thal is unknown. The identification of disease burden and modulating factors in children with HbSβ-thal may help to provide a targeted clinical approach, resulting in a better quality of life and life expectancy. In this study, the natural history of HbSβ-thal in children from Minas Gerais, a southeastern state of Brazil, is described. Additionally, we estimated the incidence of HbSβ-thal in this state.

Material and methods

Study design and setting

This was a retrospective cohort study involving children with HbSβ-thal diagnosed by the Newborn Screening Program (NSP) from Minas Gerais, Brazil. For the description of natural history, the sample consisted of children born between January 1999 and December 2015, screened by NSP and followed-up at the outpatient clinic of Hemominas, Blood Center of Minas Gerais. To study the incidence of HbSβ^0-thal and HbSβ^+-thal in Minas Gerais, all children screened by NSP in the state between January 2011 and December 2015 with a suggestive isoelectric (IEF) and HPLC profile of HbSβ-thal were included. In these cases, *HBB* sequencing was always done to confirm the genotype.

Eligibility criteria

Inclusion criteria for participants with Hb Sβ-thal were: 1) children born between January 1999 and December 2015 screened by NSP with HbSβ-thal and followed up at the Blood Center; 2) Written informed consent obtained from participants' parents or guardians and from children's assent when appropriate. Inclusion criteria to estimate the incidence of HbSβ-thal in the state were: all children born between January 2011 and December 2015 (five years) screened by NSP with HbSβ-thal, according to the previous described definition. This period limitation was necessary because allele-specific PCR reaction for codon 7 of *HBB* (GAG, GTG, or AAG) was only introduced as a NSP routine in March 2010. All babies with Hb FS or FSA detected by IEF and HPLC had, then, their *HBB* gene sequenced.

Measurements

Data collection instruments

A medical record data abstraction was performed for all consenting participants. Medical records were reviewed by hematologists or research nurses under the supervision of hematologists. Clinical, laboratory, and treatment data were abstracted using standardized definitions of the clinical outcomes of SCD[11] and entered into a specific database. The period of clinical follow-up of the study population was between 01/01/1999 (beginning of the cohort follow-up) and 01/01/2019 (end of the cohort follow-up). All children had at least 2.8 years at last clinical visit. Only clinical outcomes recorded before initiation of modifying therapies (hydroxyurea or chronic transfusion program) were considered.

Transcranial Doppler (TCD) examinations were all made and interpreted by a single expert. The Stroke Prevention Trial in the Sickle Cell Anemia study protocol[12] was followed, with pulse TCD and a 2 MHz probe for a full Doppler test (EME TC 2000; Nicolet, Madison, WI, USA).

Molecular analysis

Genomic DNA extraction from blood samples was performed using a commercial kit (QIAamp, DNA Blood Mini Kit, Qiagen, Hilden, Germany). The identification of β-Thal mutations was conducted by Sanger sequencing (Supplementary Methods).

Children were classified as having HbSβ^0-thal, severe HbSβ^+-thal, intermediate HbSβ^+-thal, and mild HbSβ^+-thal, according to β-thalassemia mutation (Table 1). The classification was based on genotype-phenotype correlations that have been shown in this and previously published studies,[3,4,13-22] as well as review of Hb variant databases.[23,24]

β^S and β^{Thal} haplotypes were determined by PCR with specific primers, followed by Restriction Fragment Length Polymorphism (RFLP) or Sanger sequencing. The primer sequences, PCR protocols, and RFLP conditions are shown in the Supplementary Table 1. Detection of *HBA* more frequent deletions ($-\alpha^{3.7}$, $-\alpha^{4.2}$, $--^{SEA}$, $--^{FIL}$, $--^{MED}$, $-(\alpha)^{20.5}$, and $-^{THAI}$) and *HBA* triplication were carried out by multiplex gap PCR as described previously[25]; the primers annealing temperature was increased to 62 °C. Identification of the Corfu

Table 1 – Classification of β-thalassemia mutations according to the clinical severity of the hemoglobinopathy Sβ-thalassemia in children.

Classification according to clinical severity	β-thalassemia mutations
Hb Sβ^0-thalassemia	IVS-I-1 G>A IVS-I-2 T>C CD39 C>T IVS-II-1 G>A IVS-II-849 A>G
Hb Sβ^+-thalassemia – severe	IVS-I-5 G>A IVS-I-110 G>A
Hb Sβ^+-thalassemia – intermediate	−29 A>G −88 C>T IVS-I-6 T>C Poly A signal – AATAAA>AACAAA
Hb Sβ^+-thalassemia – mild	−101 C>T −92 C>T IVSII-839 T>C + IVS-II-844 C>A in *cis*

deletion in participants with IVSI-5 G>A was performed by Multiplex Ligation-dependent Probe Amplification.

Laboratory data

Total Hb, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), white blood cell (WBC) count, reticulocyte, and platelet count were determined using electronic cell counter (CELL-DYN Ruby, Abbott Laboratories, Santa Clara, CA, USA). Hemoglobin fractions were quantified by high-performance liquid chromatography (Beta-thalassaemia Short Program on the Variant II analyzer, BioRad, Hercules, CA, USA). The mean of Hb, MCV, MCH, WBC, platelet, and reticulocyte counts were calculated for children above two years of age, and steady-state values were registered. Values obtained from tests performed after blood transfusion (up to 90 days) and during serious clinical illness (vaso-occlusive pain crisis, severe infection, acute splenic sequestration, and aplastic crisis) were disregarded. For children who underwent modifying therapies (hydroxyurea and/or chronic blood transfusion program), steady state values were determined from the data registered before initiating the therapy.

Statistical analysis

Continuous variables were expressed as mean and standard error of the mean (SEM) or median and interquartile range (IQ) as appropriate. Categorical variables were expressed as percentages of total and 95 % confidence interval (CI). Normal distribution of continuous variables was checked by the Kolmogorov-Smirnov test. The univariate analysis of continuous variables was performed using the unpaired *t*-test, except for those variables with deviation from normal distribution, in which case Mann-Whitney U test was used. Univariate associations between categorical variables were evaluated using Fisher's exact test. The incidence of clinical outcomes was reported by relative rates to 100 patient-years (pt-yrs) and 95 % CI. The incidence rate ratio (IRR) of clinical outcomes between groups was compared using OpenEpi online software,[26] by Fisher's Exact Test. Cumulative risk of acute splenic sequestration was estimated by using a Kaplan-Meier method [function (1− survival)] and the log rank test was used to compare different subgroups based on risk factors. Birth date determined entry into the study program. Death by any causes, and last clinical visit without acute splenic sequestration until January 2019 (end of follow-up) were reasons for censored observations. Tests with probability $p < 0.05$ were considered significant. Statistical analyses were performed with the SPSS 20.0 (SPSS Inc.; Chicago, IL, USA).

Results

Hb Sβ-thal incidence in the state of Minas Gerais

From 1,179,389 neonates born between 01-Jan-2011 and 31-Dec-2015, 53 were diagnosed with HbSβ-thal. Out of these 53 babies, 26 (49.1 %) had HbSβ^0-thal and 27 (50.9 %) HbSβ^+-Thal (all subtypes). The overall incidence of HbSβ-thal in the state was 1:22,250 (95 % CI, 1:17,530 to 1:30,450). The incidence of HbSβ^0-thal was 1:45,360 (95 % CI, 1:32,770 to 1:73,680) and that of HbSβ^+-thal was 1:43,680 (95 % CI, 1:31,720 to 1:70,140).

Cohort characteristics

From January 1999 to December 2015, 127 neonates screened with a suggestive clinical and laboratory picture of HbSβ-thal were sent to clinical follow-up at the Blood Center. Out of these 127 children, two individuals did not consent to participating in this study, one was deceased, eight were transferred to other outpatient clinics, and 27 had genotypes other than HbSβ-thal (HbSS, HbS/HPFH, HbS/Porto Alegre, HbS/Saki, HbS/Köln, HbS/Yaizu, and sickle cell trait). Therefore, 89 children with HbSβ-thal were included in the present study.

The median age at last clinical visit was 11.7 y (IQ range, 8.3 y); 52.8 % were females. The median follow-up up to starting HU or CBT was 9.8 years (IQ range: 9.0 years), providing 805.8 patient-years. Twenty-nine (32.6 %) children received hydroxyurea (HU) therapy and three (3.4 %), blood transfusion (CBT) and HU during follow-up. There were 14 different types of β-thal causative variants (Figure 1). The Corfu delta-beta thalassemia deletion was not detected in any of the five families with IVS-I-5 G>A mutation (NG_000007.3: g.57237_64443del7207).

Clinical profile of children with HbSβ-thalassemia

Including all children, 43 (48 %) had the spleen border at least 3 cm below the left costal margin. Out of these, 30 (69.8 %) had HbSβ^0-thal and 13 (30.2 %), HbSβ^+-thal (seven in eight children with IVS-I-110 G>A, four in ten with IVS-I-6 T>C, and two in six children with IVS-I-5 G>A mutations). Hypersplenism was observed in three children. Thirty-three children (37.1 %) had at least one episode of acute splenic sequestration (ASS). Age at first acute splenic sequestration ranged from 5 months to

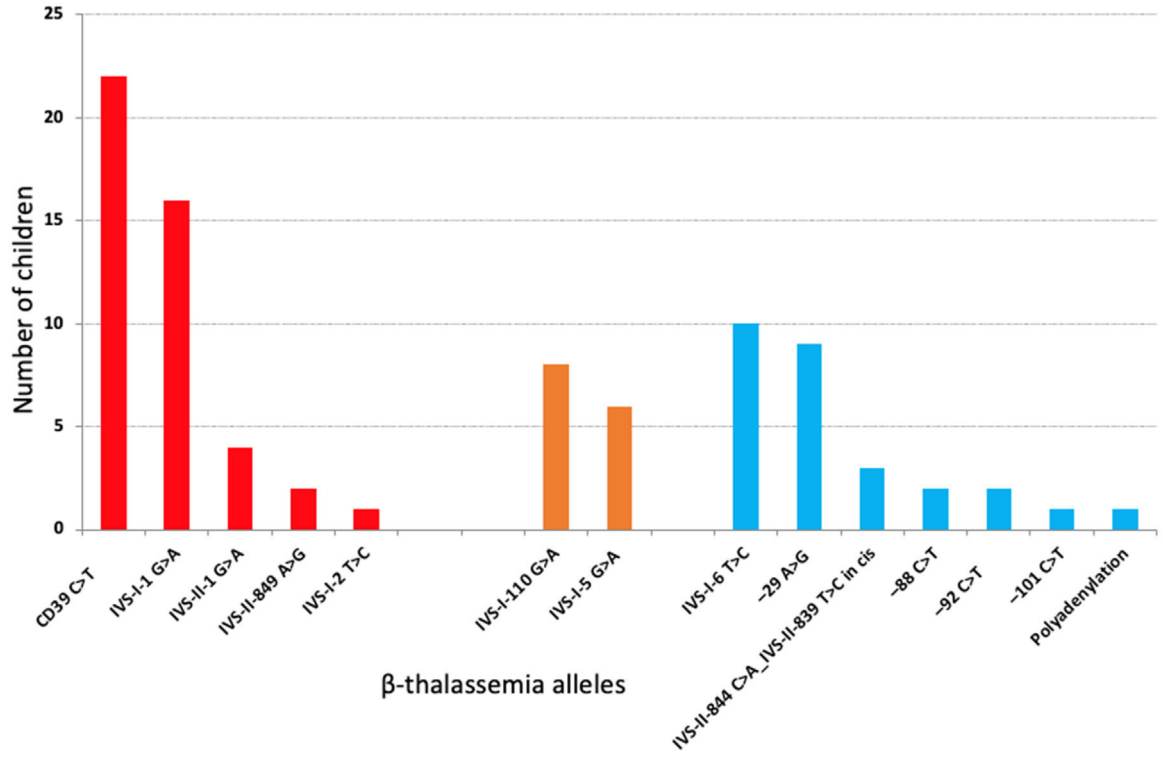

Figure 1 – Frequency of 14 different types of β-thal causative variants in 87 children with hemoglobinopathy S/beta-thalassemia. In two children, sequencing did not disclose any mutation. In red, S/beta^0-thalassemia; in orange, severe S/beta$^+$-thalassemia; in blue, intermediate or mild S/beta$^+$-thalassemia.

7.6 years, and the cumulative probability was 41.2 % ± 5.7 %. Out of those 33 children, 25 (75.8 %) had HbSβ^0-thal and eight (24.2 %), HbSβ^+-thal (4/8 with IVS-I-110 G>A, 2/6 with IVS-I-5 G>A, and 2/10 with IVS-I-6 T>C, and mutations). Adding HbSβ^0-thal to severe HbSβ^+-thal and comparing with moderate and mild HbSβ^+-thal, the cumulative probabilities of ASS were 59.3 %±7.2 % and 7.8 %±5.3 %, respectively (p = 0.00005; Figure 2).

Seventy-seven (85 %) children had at least one acute vaso-occlusive crisis resulting in pain (VOCP), totaling 647 events during follow-up period. The incidence of VOCP was 83.6 events per 100 pt-yrs (95 % CI: 77.4 – 90.2). During the follow-up, 82 (94.3 %) children had at least one infection episode requiring antibiotic administration. The incidence of infection was 79 events per 100 pt-yrs (95 % CI: 72.9 – 85.4). Forty-eight (55.2 %) children had at least one episode of acute chest syndrome (ACS), as defined in [11]. The incidence of ACS was 14.5 events per 100 pt-yrs (95 % CI: 12.0 – 17.4). At least one red blood cell transfusion was necessary in 49 children (56.3 %). The incidence of transfusion was 14.5 events per 100 pt-yrs (95 % CI: 12.0 – 17.4). Incidence of clinical events of HbSβ-thal children according to β-thalassemia causative variants is depicted in Table 2.

Out of 89 HbSβ-thal children, one (1.1 %) had already had stroke (CD39 C>T) before starting the TCD screening in our center. Out of those 89, 68 (76.4 %) had at least one TCD test. Two children (IVS-I-5 G>A and IVS-I-110 G>A) had low conditional TCD and 66 (97.1 %) had low-risk TCD.

Laboratory profile of children with HbSβ-thalassemia

In general, children with HbSβ-thal had low levels of MCH and MCV while HbA2 levels were high; there was no significant difference in levels of MCH, MCV, and HbA2 levels between individuals with HbSβ^0-thal and HbSβ^+-thal. Children with HbSβ^0-thal had significantly lower total Hb levels and higher WBC, platelets, reticulocytes, and HbF when compared to those with HbSβ^+-thal. Children with HbSβ^+-thal presented a wide spectrum of HbA, ranging from 3.7 % (IVS-I-5 G>A) to 45.2 % (−92 C>T). Laboratory data of HbSβ-thal children according to β-thalassemia mutation are depicted in Table 3.

Effect of β-thalassemia mutation

Clinical and laboratory characteristics of patients according to beta thalassemia mutation are depicted in Tables 2 and 3, respectively. Laboratory profile was similar in children with CD39 C>T, IVS-I-1 G>A, IVS-I-5 G>A, or IVS-I-110 G>A causative variants, except for HbA2, and the degree of microcytosis and hypochromia (Supplementary Table 2). However, children with IVS-I-1 G>A mutation had significantly higher incidence of infection and ACS when compared to those with CD39 C>T (p<0.01), while children with HbSβ^+-severe (IVS-I-110 G>A or IVS-I-5 G>A) had similar incidence of clinical outcomes when compared to those with CD39 C>T (p>0.05).

Children with CD39 C>T had a more severe laboratory profile when compared with those with −29 A>G or IVS-I-6 T>C,

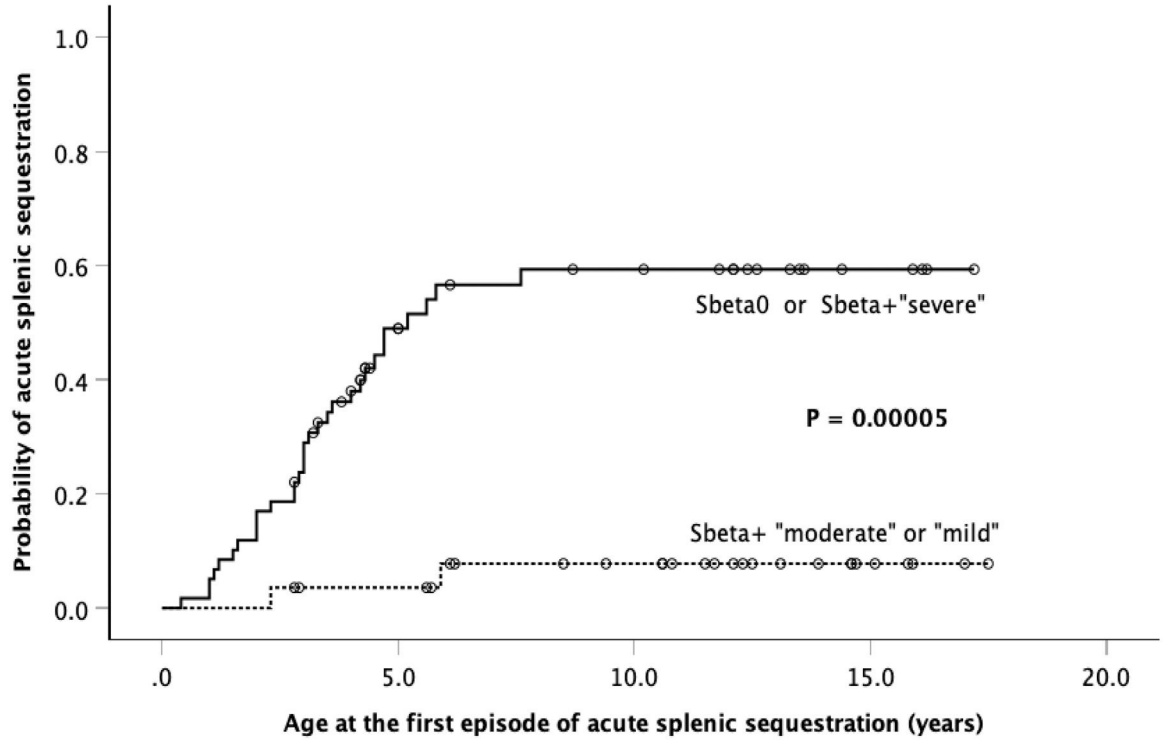

Figure 2 – Kaplan-Meier probability curve [Plot (1-Survival)] for the occurrence of the first episode of acute splenic sequestration comparing children with "HbSβ^0 or severe HbSβ$^+$-thalassemia" and those with "moderate or mild HbSβ$^+$-thalassemia".

except for MCV and MCH values that were similar (Supplementary Table 3). Furthermore, children with −29 A>G or IVS I-6 T>C variants presented significantly lower incidence of infection and transfusion when compared to those with CD39 C>T (p<0.01), while children with −29 A>G also had significantly lower incidence of VOCP when compared to those with CD39 C>T (p<0.01).

Children with HbSβ^+-intermediate had a more severe laboratory profile when compared with those with HbSβ^+-mild (Supplementary Table 4). Furthermore, children with HbSβ^+-intermediate had significantly higher incidence of infections (p = 0.001), VOCP (p<0.001), and ACS (p = 0.046) when compared to those with HbSβ^+-mild. However, there was no significant difference in the incidence of transfusions between

Table 2 – Incidence of clinical events per 100 patient-years in children with HbSβ-thalassemia according to β-thalassemia mutation.

Beta-thalassemia mutation	Infection requiring antibiotics[a]	Acute pain from tissue ischemia[a]	Acute chest syndrome[a]	Red Blood Cell Transfusion[a]
−101 C>T (1)	12.7 (1.54–45.8)	12.7 (1.54–45.8)	0	0
−92 C>T (2)	34.6 (14.9–68.2)	13 (2.68–37.9)	0	0
−88 C>T (2)	18.2 (5.9–42.4)	3.63 (0.09–20.3)	0	0
−29 A>G (9)	54 (39.4–72.3)	32.4 (21.4–47.2)	8.4 (3.4–17.3)	1.2 (0.03–6.7)
IVS-I-1 G>A (16)	122.6 (105.1–142.6)	114.6 (97.3–133.9)	40.1 (30.2–52.2)	44.5 (34.1–57.2)
IVS-I-2 T>C (1)	94.8 (47.3–169.7)	86.2 (41.3–158.5)	25.9 (5.3–75.6)	60.3 (24.3–124.3)
IVS-I-5 G>A (6)	105 (68–155.1)	137.5 (94–194.1)	25.8 (9.5–56.1)	12.6 (2.6–36.8)
IVS-I-6 T>C (10)	61.6 (48.8–77.2)	114.2 (96–134.9)	7.4 (3.4–14)	3.3 (1–7.9)
IVS-I-110 G>A (8)	109.2 (85.8–137.1)	73.8 (54.8–97.3)	8.9 (3.3–19.3)	32.5 (20.4–49.2)
CD39 C>T (22)	89.6 (76.9–103.9)	97.2 (84–112)	10.6 (6.6–16.3)	35.4 (27.6–44.8)
IVS-II-1 G>A (4)	54.7 (34.7–82.1)	45.2 (27.2–70.6)	11.9 (3.9–27.8)	33.3 (18.2–55.9)
IVS-II-839 T>C and II-844 C>A (3) *in cis*	18.9 (6.9–44.1)	11.3 (2.3–33.1)	0	0
IVS-II-849 A>G (2)	107.4 (51.5–197.5)	139.6 (74.4–23.9)	42.9 (11.7–110)	0
Poly-A signal (1)	27.6 (8.8–66.6)	148.1 (91.7–226.4)	0	13.8 (1.7–49.9)

[a] Incidence per 100 patient-years and 95 % confidence interval. Definition of clinical events according to.[11]
Data for groups with less than 5 children should be interpreted with caution.

Table 3 – Baseline laboratory data of children with HbSβ-thalassemia according to β-thalassemia mutation.

Beta-thalassemia mutation (n)	Hb Total	HbA (%)	HbF (%)	HbA2 (%)	Retic (%)	WBC(x10^9/L)	Platelets(x10^9/L)	MCV(fL)	MCH (pg)
−101 C>T (1)	13.6	45.1	1.3	4.7	0.9	6.33	246.8	79.1	26.4
−92 C>T (2)	12.25±0.55	44.3 ± 1.0	1.9 ± 0.6	4.3 ± 0.2	0.9 ± 0.1	7.69±1.92	285.31±17.11	78.5 ± 0.4	25.7 ± 0.1
−88 C>T (2)	12.05±0.35	20.1 ± 5.0	19.9 ± 7.1	5.8 ± 1.1	1.8 ± 0.0	7.65±0.24	271.30±19.91	72.8 ± 2.6	26.1 ± 1.7
−29 A>G (9)	11.37±0.27	20.5 ± 0.7	7.8 ± 1.1	5.9 ± 1.1	2.7 ± 0.4	8.81±0.78	274.91±17.26	73.1 ± 1.7	23.5 ± 0.6
IVS-I-1 G>A (16)	7.80±0.20	0.0	11.4 ± 2.1	6.1 ± 1.0	12.4 ± 1.1	13.28±0.91	398.57±27.16	71.0 ± 1.2	21.5 ± 0.5
IVS-I-2 T>C (1)	7.7	0.0	21.0	4.0	11.90	9.2	403.4	75.0	22.9
IVS-I-5 G>A (6)	8.62±0.49	4.1 ± 0.17*	16.9 ± 2.7*	5.8 ± 0.76*	10.3 ± 1.9	15.22±2.05	380.62±60.51	68.8 ± 1.0	20.5 ± 0.7
IVS-I-6 T>C (10)	10.44±0.21	26.3 ± 0.4	3.0 ± 0.7	4.8 ± 0.8	3.4 ± 0.6	8.53±0.60	265.24±20.76	69.5 ± 1.7	22.0 ± 0.5
IVS-I-110 G>A (8)	8.28±0.57	11.2 ± 0.7	9.8 ± 3.0	6.1 ± 0.7	10.3 ± 1.9	14.81±1.38	324.61±41.03	69.7 ± 1.8	22.0 ± 0.5
CD39 C>T (22)	8.09±0.17	0.0	15.5 ± 1.7	5.1 ± 1.1	11.4 ± 0.8	11.69±0.64	355.45±27.85	74.5 ± 1.5	23.2 ± 0.5
IVS-II-1 G>A (4)	8.80±0.19	0.0	19.6 ± 1.4	4.0 ± 1.2	9.7 ± 2.1	11.34±0.82	307.63±35.67	73.0 ± 2.1	22.6 ± 0.8
IVS-II-839 T>C and II-844 C>A (3) *in cis*	12.77±0.34	44.2 ± 0.6	1.5 ± 0.5	4.3 ± 0.2	1.4 ± 0.2	7.59±1.53	376.84±72.57	76.5 ± 1.8	24.4 ± 0.6
IVS-II-849 A>G (2)	9.15±0.15	0.0	13.8 ± 0.8	5.5 ± 0.7	4.4 ± 0.6	11.46±0.14	445.22±40.92	64.9 ± 3.6	19.4 ± 1.3
Poly-A signal (1)	9.2	16.4	9.8	5.0	6.6	6.07	190.59	71.9	21.7

Data shown as mean ± standard error of the mean (±SEM).
Data for groups with less than 5 children should be interpreted with caution.
* These values were calculated as the mean (±SEM) for five children who were on hydroxyurea (HPLC method, Trinity Biotech Premier Resolution system).

individuals with HbSβ^+-intermediate and HbSβ^+-mild ($p = 0.39$; Supplementary Table 4).

Effect of α-thalassemia

The quantity or quality of genomic DNA was not sufficient for genotyping haplotypes and α-thal in one child (−29 A>G). About a fifth ($n = 18/86$; 20.9 %) of the children had coexistent a 3.7 kb *HBA1/HBA2* deletion (16 $\alpha\alpha/\text{-}\alpha^{3.7}$, one $\text{-}\alpha^{3.7}/\text{-}\alpha^{3.7}$, and one $\alpha\alpha/\alpha\alpha\alpha^{-3.7}$). The analysis to assess the effect of α-thal on phenotype was restricted to children with the mutations CD39 C>T or IVS I-1 G>A because there was a higher number of individuals in these groups. There were no significant association between laboratory and clinical characteristics and coexistence of 3.7 kb *HBA1/HBA2* deletion (data not shown).

Effect of beta-globin gene cluster haplotypes

Frequency of β^S- and β^{Thal}-haplotypes according to β-thalassemia mutation in 86 children with HbSβ-thalassemia is depicted in Table 4. The analysis to assess the effect of β^S-haplotypes on phenotype was restricted to children with the mutations CD39 C>T. Children with the Benin haplotype had a higher incidence of infections and ACS than children with CAR ($p = 0.04$ and $p = 0.03$, respectively). There was no association between β^S-haplotypes and transfusions or acute splenic sequestration in children with CD39 C>T.

Table 4 – Frequency of β^S and β^{Thal}-haplotypes according to β-thalassemia mutation in 86 children with HbSβ-thalassemia.

β-thalassemia mutation (n)	β^S/β^{Thal}-haplotypes (n)
−101 C>T (1)	Ben/I (1)
−92 C>T (2)	CAR/V (2)
−88 C>T (2)	CAR/VII (1) and CAR/ATP (1)
−29 A>G TATA box (8)	Ben/IX (3), Sen/IX (2), Car/IX (1), CAR/I (1), Ben/ATP (1)
IVS-I-1 G>A (16)	CAR/V (8), Ben/V (6), CAR/I (2)
IVS-I-2 T>C (1)	CAR/ATP or Ben/ATP
IVS-I-5 G>A (6)	CAR/V (4, two siblings), Ben/V (1), ATP/V (1)
IVS-I-6 T>C (10)	CAR/VI (2), Ben/VII (2), CAR/IV (1), Ben/VI (1), CAR/?[a] (4)
IVS-I-110 G>A (8)	CAR/I (5), Ben/I (2), Ben/?[a] (1)
CD39 C>T (22)	CAR/II (11), Ben/II (2), CAR/?[a] (5), Ben/?[a] (4)
IVS-II-1 G>A (4)	CAR/V (1), Ben/V (1), CAR/ATP or Ben/APT (2 siblings)
IVS-II-839 T>C and IVS-II-844 C>A in *cis* (3)	CAR/ATP or Ben/ATP (3)
IVS-II-849 A>G (2)	CAR/ATP or Ben/ATP (2 siblings)
Poly-A signal (1)	CAR/I (1)

[a] β^{Thal}-haplotype could not be defined.

Discussion

The relative incidence of HbSβ-thal represents about 6 % of all cases with SCD in Minas Gerais. The incidence of HbSβ^+-thal and that of HbSβ^0-thal subtypes were almost the same.

To the best of our knowledge, the state of Minas Gerais is the most heterogeneous state in terms of β-Thal alleles in Brazil. The most prevalent allele found in the present study was CD39 C>T, corroborating previous studies carried out in the Southeastern region of Brazil.[13,27-34] In the Mediterranean population, this variant accounts for the second most common one.[4]

In the present cohort, children with HbSβ^+-thal presented a broad spectrum of clinical phenotypes, while children with HbSβ^0-thal generally had a severe disease, as demonstrated by several reports.[13]

Overall, 14 different β-thalassemia mutations were identified in this study. While the type of β-thalassemia mutation clearly affected the clinical outcome of children with HbSβ-

thal, co-inherited α-thal and β^S-haplotypes had no clinical impact in the present study. The relative HbA concentration has a protective effect on the morbidity of HbSβ-thal, most probably due to decrease in polymerization, sickling of red blood cells and other subsequent pathophysiological effects.[35]

The mutations found in the present study were categorized into four distinct groups according to clinical severity. The first comprises all genotypes without HbA production (HbSβ^0-thal). Individuals with the HbSβ^+-thal genotype were categorized into severe, moderate, and mild forms. Individuals with the IVSI-110 G>A or IVS-I-5 G>A mutations, with a relative concentration of HbA lower than 10 %, were included in the group of severe HbSβ^+ and had, particularly those with IVS-I-5 G>A,[36] clinical features similar to those with HbSβ^0-thal.[13] Individuals with mutations - 29 A>G, −88 C>T, IVS-I-6 T>C and in the polyadenylation region, with HbA concentrations between 16 % and 26 %, were included in the moderate HbSβ^+ group. Children with −101 C>T, −92 C>T, and IVS-II double mutation *in cis* had HbA above 40 % and a very mild disease.

In this study, the prevalence of splenomegaly was 31 %, similar to other previous studies.[5,37] Prevalence of splenomegaly was higher in children with the HbSβ^0-thal and severe HbSβ^+-thal (group 1) when compared to those with moderate or mild HbSβ^+-thal (group 2). Correspondingly, group 1 had more acute splenic sequestration than group 2. Therefore, low HbA levels in the severe forms of HbSβ^+-thal do not exempt these patients from having splenic events similar to those with HbSβ^0-thal. This finding may be used to target those individuals at highest risk for the occurrence of splenic crises in educational and prevention policies for SCD.

Evaluation of clinical manifestations in children with different β-Thal mutations generally revealed that the incidence of clinical events per 100 patient-years was similar in those with HbSβ^0-Thal and with IVS-I-5 G>A or IVS-I-110 G>A genotypes. These data can be explained by the low concentration of HbA observed in both β^+-Thal mutations, as observed by others[38,39] and by our group.[36]

In general, the clinical picture of HbSβ^0-thal children with different mutations was similar, as expected from the absence of Hb A production. When they are compared to homozygous Hb SS patients, similarities are prominent[40] and traditionally Hb SS and HbSβ^0-thal are merged into a clinical entity named sickle cell anemia. However, differences do exist. For instance, cerebrovascular events are much less common in children with HbSβ^0-thal when compared to those with Hb SS.[41] Only a single case of overt stroke in a child with CD39 C>T was observed in the present cohort. As stated in Results, mild abnormalities in TCD were present in only two children with severe HbSβ^+-thal.

Although the −29 TATA box mutation is often related to general mild clinical presentation, it has been associated in other studies with more severe clinical outcomes related to blood viscosity.[3,4] Although some important differences in the studies hinder direct comparisons, our data corroborate these previous studies since VOCP and ACS were common clinical manifestations observed in children with −29 TATA box. Out of nine children with this mutation, five (55.6 %) had at least one ACS and an average of 2 ± 3.4 episodes of ACS was recorded in the follow-up period. These findings are important to guide clinical management in patients with this allele.

The effects of co-inheritance of α-thal in individuals with HbSβ-thal are still not well understood and there are controversies in the literature. Some studies showed a strong modulation of α-thal in HbSβ-thal phenotype,[42,43] while other studies did not find significant influence.[10,39,44] We have previously observed influence of α-thal in clinical and laboratory characteristics of children with HbSS and HbSC,[8,9] suggesting an impact of α-thal in morbidity of SCD in our population. However, co-inheritance of α-thal had no significant influence on the laboratory and clinical parameters of children with the most frequent mutations in this study (IVS I-1 G>A and CD 39 C>T). Further studies are thus needed to understand the influence of co-inheritance of α-thal on the phenotypes of individuals with HbSβ-thal.

During Brazil colonization, the main people brought by slave ships came from the regions of Bantu (CAR) and Benin.[37] This is corroborated with higher frequency of the CAR (57.5 %) and Benin (28.7 %) β^S-haplotypes observed in this study. In addition, other studies carried out in the state of Minas Gerais also detected a high frequency of the CAR and Benin haplotypes in individuals with HbSS and HbSC.[8,41] β^S-haplotypes were postulated to influence the severity of SCD by influencing the concentration of HbF. However, there is no evidence to suggest that β^S-haplotypes give any more information than measuring the HbF level.[7] Although we observed a higher incidence of infections and ACS in children with Benin β^S-haplotype when compared to those with CAR, the clinical impact is not relevant, making it unfeasible to use the β^S-haplotypes as a marker for HbSβ-thal severity. Our findings do not support the hypothesis that the CAR β^S-haplotype leads to a more severe phenotype. We previously observed no difference in the phenotypic characteristics of individuals with the CAR and Benin haplotypes in our cohorts of HbSS and HbSC children.[8,41]

The most frequent β^{thal}-haplotypes in the present study were V (27.6 %), I (14.9 %) and II (14.9 %), and were identified mainly with β-thal alelles of European origin, such as IVS-I-110 G >A and CD39 C>T. However, some β^{thal}-haplotypes found in this study are from African origin, such as those identified in the −29 C>T and in −88 C>T mutations. β^{thal}-haplotypes are not restricted to a single β-thal mutation and have a heterogeneous distribution.[45].

There are some limitations of this study. First, it has a retrospective design and missing data in medical records are possible. Second, given that the participants are children, long-term complications were not evaluated. Finally, the low frequency of some β-thal mutations as well as the low frequency of β^S-haplotypes and α-thalassemia within each β-thal mutation are limiting factors for a more accurate evaluation of genotype-phenotype correlation.

Conclusions

The incidence of HbSβ-thal in the Brazilian state of Minas Gerais is 1 per 22,250 newborns. Laboratory parameters and incidence of clinical manifestations are quite similar in children with HbSβ^0-thal genotypes and severe HbSβ^+-thal, while infants with HbSβ^+-thal intermediate or mild had a varying clinical presentation. We have shown that the underlying

β-thalassemia mutation was associated with clinical and laboratory features in children with HbSβ-thal, while β^S-haplotypes and α-thalassemia did not meaningfully influence the phenotype. The early identification of β-thalassemia mutation may help the clinical management of children and lead to a better prognosis and survival of children with HbSβ-thal.

Conflicts of interest

All authors declare that they have no conflict of interest.

Funding

This work was supported by a grant from the "Fundação de Amparo à Pesquisa de Minas Gerais" (FAPEMIG; grant # APQ-00131-17); Marcos Borato Viana is a researcher from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico"); award number 312112/2020-3.

Ethical approval

The study protocol was approved by institutional review boards and national research committee and all procedures were in accordance with the 1964 Helsinki declaration and its later amendments.

Informed consent

Informed written consents were obtained from participants' parents or guardians and the children's assent was obtained, when appropriate.

Contribution of the authors

Érica Louback de Oliveira: Conceptualization, Methodology, Data curation, Writing - Original draft preparation, reviewing; André Rolim Belisário: Conceptualizaton, Methodology, Data curation, Project administration, Writing - Original draft preparation, reviewing; Natiely Pereira Silva: Methodology, Data curation, Writing - reviewing; Paulo do Val Rezende: Clinical Follow-up, Data abstraction, Writing – reviewing; Maristela Braga Muniz: Data abstraction, Data curation, Writing – reviewing; Larissa Maira Moura Oliveira: Methodology, Data curation, Writing - reviewing; Cibele Velloso-Rodrigues: Conceptualization, Writing - draft preparation, reviewing; Marcos Borato Viana: Conceptualization, Supervision, Formal analysis, Writing - draft preparation, reviewing and editing, Funding acquisition.

Acknowledgments

The authors acknowledge all participants and parents for their cooperation in being part of this study. We also thank the hematology laboratory of the State University of Campinas (UNICAMP), and particularly Prof. Maria de Fátima Sonati, for testing the Corfu delta-beta thalassemia deletion.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.htct.2023.11.002.

REFERENCES

1. Kato GJ, Piel FB, Reid CD, Gaston MH, Ohene-Frempong K, Krishnamurti L, Smith WR, Panepinto JA, Weatherall DJ, Costa FF, Vichinsky EP. Sickle cell disease. Nat Rev Dis Primers. 2018;4:18010. https://doi.org/10.1038/nrdp.2018.10.
2. Cançado R., Jesus J.A. Sickle cell disease in Brazil. Rev Bras Hematol Hemoter. 2007;29(3):204–6. https://doi.org/10.1590/S1516-84842007000300002.
3. Serjeant GR, Serjeant BE, Fraser RA, Hambleton IR, Higgs DR, Kulozik AE, et al. Hb S-beta-thalassemia: molecular, hematological and clinical comparisons. Hemoglobin. 2011;35(1):1–12. https://doi.org/10.3109/03630269.2010.546306.
4. Thein SL. The molecular basis of beta-thalassemia. Cold Spring Harb Perspect Med. 2013;3(5):a011700. https://doi.org/10.1101/cshperspect.a011700.
5. Serjeant GR, Sommereux AM, Stevenson M, Mason K, Serjeant BE. Comparison of sickle cell-beta0 thalassaemia with homozygous sickle cell disease. Br J Haematol. 1979;41(1):83–93. https://doi.org/10.1111/j.1365-2141.1979.tb03684.x.
6. Benites BD, Bastos SO, Baldanzi G, Dos Santos AO, Ramos CD, Costa FF, et al. Sickle cell/beta-thalassemia: comparison of Sbeta0 and Sbeta+ Brazilian patients followed at a single institution. Hematology. 2016: 1–7. https://doi.org/10.1080/10245332.2016.1187843.
7. Rees DC, Brousse VAM, Brewin JN. Determinants of severity in sickle cell disease. Blood Rev. 2022;56:100983. https://doi.org/10.1016/j.blre.2022.100983.
8. Belisario AR, Rodrigues CV, Martins ML, Silva CM, Viana MB. Coinheritance of alpha-thalassemia decreases the risk of cerebrovascular disease in a cohort of children with sickle cell anemia. Hemoglobin. 2010;34(6):516–29. https://doi.org/10.3109/03630269.2010.526003.
9. Rezende PV, Belisario AR, Oliveira EL, Almeida JA, Oliveira LMM, Muniz M, et al. Co-inheritance of a-thalassemia dramatically decreases the risk of acute splenic sequestration in a large cohort of newborns with hemoglobin SC. Haematologica. 2019;104(7):e281–e3. https://doi.org/10.3324/haematol.2018.209221.
10. Steinberg MH, Coleman MB, Adams JG, Rosenstock W. Interaction between HBS-beta-o-thalassemia and alpha-thalassemia. Am J Med Sci. 1984;288(5):195–9. https://doi.org/10.1097/00000441-198412000-00001.
11. Ballas SK, Lieff S, Benjamin LJ, Dampier CD, Heeney MM, Hoppe C, et al. Definitions of the phenotypic manifestations of sickle cell disease. Am J Hematol. 2010;85(1):6–13. https://doi.org/10.1002/ajh.21550.
12. Adams RJ, McKie VC, Hsu L, Files B, Vichinsky E, Pegelow C, et al. Prevention of a first stroke by transfusions in children with sickle cell anemia and abnormal results on transcranial Doppler ultrasonography. N Engl J Med. 1998;339(1):5–11. https://doi.org/10.1056/NEJM199807023390102.
13. Belisario AR, Carneiro-Proietti AB, Sabino EC, Araujo A, Loureiro P, Maximo C, et al. Hb S/beta-thalassemia in the REDS-III Brazil sickle cell disease cohort: clinical, laboratory and molecular characteristics. Hemoglobin. 2020: 1–9. https://doi.org/10.1080/03630269.2020.1731530.

14. Treisman R, Orkin SH, Maniatis T. Specific transcription and RNA splicing defects in five cloned beta-thalassaemia genes. Nature. 1983;302(5909):591–6. https://doi.org/10.1038/302591a0.
15. Antonarakis SE, Irkin SH, Cheng TC, Scott AF, Sexton JP, Trusko SP, et al. beta-Thalassemia in American Blacks: novel mutations in the "TATA" box and an acceptor splice site. P Natl Acad Sci USA. 1984;81(4):1154–8. https://doi.org/10.1073/pnas.81.4.1154.
16. Atweh GF, Wong C, Reed R, Antonarakis SE, Zhu D, Ghosh PK, et al. A new mutation in IVS-1 of the human beta globin gene causing beta thalassemia due to abnormal splicing. Blood. 1987;70(1):147–51. PMID: 2439149.
17. Christakis J, Vavatsi N, Hassapopoulou H, Angeloudi M, Papadopoulou M, Loukopoulos D, et al. A comparison of sickle cell syndromes in northern Greece. Br J Haematol. 1991;77(3):386–91. https://doi.org/10.1111/j.1365-2141.1991.tb08589.x.
18. Kulozik AE, Bail S, Kar BC, Serjeant BE, Serjeant GE. Sickle cell-beta+ thalassaemia in Orissa State, India. Br J Haematol. 1991;77(2):215–20. https://doi.org/10.1111/j.1365-2141.1991.tb07980.x.
19. Rosatelli MC, Pischedda A, Meloni A, Saba L, Pomo A, Travi M, et al. Homozygous beta-thalassaemia resulting in the beta-thalassaemia carrier state phenotype. Br J Haematol. 1994;88(3):562–5. https://doi.org/10.1111/j.1365-2141.1994.tb05074.x.
20. Rund D, Cohen T, Filon D, Dowling CE, Warren TC, Barak I, et al. Evolution of a genetic disease in an ethnic isolate: beta-thalassemia in the Jews of Kurdistan. P Natl Acad Sci USA. 1991;88(1):310–4. https://doi.org/10.1073/pnas.88.1.310.
21. Goldsmith ME, Humphries RK, Ley T, Cline A, Kantor JA, Nienhuis AW. Silent" nucleotide substitution in a beta+-thalassemia globin gene activates splice site in coding sequence RNA. P Natl Acad Sci USA. 1983;80(8):2318–22. https://doi.org/10.1073/pnas.80.8.2318.
22. Humphries RK, Ley T, Goldsmith ME, Kantor JA, Cline AC, Nienhuis AW. Silent" nucleotide substitution in codon 24 of a beta+ thalassemia globin gene activates splice site in coding sequence RNA. Prog Clin Biol Res. 1983;134:123–6. PMID: 6664994.
23. Hardison RC, Chui DH, Riemer C, Giardine B, Lehvaslaiho H, Wajcman H, et al. Databases of human hemoglobin variants and other resources at the globin gene server. Hemoglobin. 2001;25(2):183–93. https://doi.org/10.1081/hem-100104027.
24. Kountouris P, Lederer CW, Fanis P, Feleki X, Old J, Kleanthous M. IthaGenes: an interactive database for haemoglobin variations and epidemiology. PLoS One. 2014;9(7):e103020. https://doi.org/10.1371/journal.pone.0103020.
25. Tan AS, Quah TC, Low PS, Chong SS. A rapid and reliable 7-deletion multiplex polymerase chain reaction assay for alpha-thalassemia. Blood. 2001;98(1):250–1. https://doi.org/10.1182/blood.V98.1.250.
26. Sullivan KM, Dean A, Soe MM. OpenEpi: a web-based epidemiologic and statistical calculator for public health. Public Health Rep. 2009;124(3):471–4. https://doi.org/10.1177/003335490912400320.
27. Fonseca SF, Kerbauy J, Escrivao C, Figueiredo MS, Cancado R, Arruda VR, et al. Genetic analysis of beta-thalassemia major and beta-thalassemia intermedia in Brazil. Hemoglobin. 1998;22(3):197–207. https://doi.org/10.3109/03630269809113134.
28. Martins CS, Ramalho AS, Sonati MF, Goncalves MS, Costa FF. Molecular characterisation of beta thalassaemia heterozygotes in Brazil. J Med Genet. 1993;30(9):797–8. https://doi.org/10.1136/jmg.30.9.797-b.
29. Araujo AS, Silva l W, Leao SA, Bandeira FC, Petrou M, Modell B, et al. A different molecular pattern of beta-thalassemia mutations in northeast Brazil. Hemoglobin. 2003;27(4):211–7. https://doi.org/10.1081/hem-120026045.
30. Reichert VC, de Castro SM, Wagner SC, de Albuquerque DM, Hutz MH, Leistner-Segal S. Identification of beta thalassemia mutations in South Brazilians. Ann Hematol. 2008;87(5):381–4. https://doi.org/10.1007/s00277-007-0418-z.
31. Zamaro PJA, Bonini-Domingos CR. The identification of beta-thalassemia mutants in Brazilians with high Hb F levels. Rev Brasileide Hematologia e Hemoterapia. 2010;32(3):215–8. https://doi.org/10.1590/S1516-84842010005000082.
32. da Silveira ZM, das Vitorias Barbosa M, de Medeiros Fernandes TA, Kimura EM, Costa FF, de Fatima, Sonati M, et al. Characterization of beta-thalassemia mutations in patients from the state of Rio Grande do Norte, Brazil. Genet Mol Biol. 2011;34(3):425–8. https://doi.org/10.1590/S1415-47572011005000032.
33. Fonseca SF, Moura Neto JP, Goncalves MS. Prevalence and molecular characterization of beta-thalassemia in the state of Bahia, Brazil: first identification of mutation HBB: c.135delC in Brazil. Hemoglobin. 2013;37(3):285–90. https://doi.org/10.3109/03630269.2013.771271.
34. Silva AN, Cardoso GL, Cunha DA, Diniz IG, Santos SE, Andrade GB, et al. The spectrum of beta-thalassemia mutations in a population from the Brazilian Amazon. Hemoglobin. 2016;40(1):20–4. https://doi.org/10.3109/03630269.2015.1083443.
35. Figueiredo MS. The compound state: Hb S/beta-thalassemia. Rev Bras Hematol Hemoter. 2015;37(3):150–2. https://doi.org/10.1016/j.bjhh.2015.02.008.
36. Viana MB, Oliveira EL, Belisario AR. Severe clinical picture in a cohort of six Brazilian children with hemoglobin Sbeta-thalassemia IVS-I-5 G>A. Blood Cells Mol Dis. 2023;104:102795. https://doi.org/10.1016/j.bcmd.2023.102795.
37. Zago MA, Costa FF, Freitas TC, Bottura C. Clinical, hematological and genetic features of sickle-cell anemia and sickle cell-beta thalassemia in a Brazilian population. Clin Genet. 1980;18(1):58–64. https://doi.org/10.1111/j.1399-0004.1980.tb01366.x.
38. Boletini E, Svobodova M, Divoky V, Baysal E, Curuk MA, Dimovski AJ, et al. Sickle cell anemia, sickle cell beta-thalassemia, and thalassemia major in Albania: characterization of mutations. Hum Genet. 1994;93(2):182–7. https://doi.org/10.1007/BF00210607.
39. Adekile AD, Akbulut N, Azab AF, Al-Sharida S, Thomas D. The sickle beta-thalassemia phenotype. J Pediatr Hematol Oncol. 2017;39(5):327–31. https://doi.org/10.1097/MPH.0000000000000747.
40. Adekile AD, Al-Sherida S, Marouf R, Mustafa N, Thomas D. The sub-phenotypes of sickle cell disease in Kuwait. Hemoglobin. 2019;43(2):83–7. https://doi.org/10.1080/03630269.2019.1610427.
41. Belisario AR, Nogueira FL, Rodrigues RS, Toledo NE, Cattabriga AL, Velloso-Rodrigues C, et al. Association of alpha-thalassemia, TNF-alpha (-308G>A) and VCAM-1 (c.1238G>C) gene polymorphisms with cerebrovascular disease in a newborn cohort of 411 children with sickle cell anemia. Blood Cells Mol Dis. 2015;54(1):44–50. https://doi.org/10.1016/j.bcmd.2014.08.001.
42. Atweh GF, Forget BG. Clinical and molecular correlations in the sickle/beta+-thalassemia syndrome. Am J Hematol. 1987;24(1):31–6. https://doi.org/10.1002/ajh.2830240105.
43. Mukherjee MB, Nadkarni AH, Gorakshakar AC, Ghosh K, Mohanty D, Colah RB. Clinical, hematologic and molecular variability of sickle cell-beta thalassemia in western India. Indian J Hum Genet. 2010;16(3):154–8. https://doi.org/10.4103/0971-6866.73410.
44. Traeger-Synodinos J, Kanavakis E, Vrettou C, Maragoudaki E, Michael T, Metaxotou-Mavromati A, et al. The triplicated alpha-globin gene locus in beta-thalassaemia heterozygotes: clinical, haematological, biosynthetic and molecular studies. Br J Haematol. 1996;95(3):467–71. https://doi.org/10.1046/j.1365-2141.1996.d01-1939.x.
45. Orkin SH, Kazazian Jr. HH, Antonarakis SE, Goff SC, Boehm CD, Sexton JP, et al. Linkage of beta-thalassaemia mutations and beta-globin gene polymorphisms with DNA polymorphisms in human beta-globin gene cluster. Nature. 1982;296(5858):627–31. https://doi.org/10.1038/296627a0.