Sickle Cell Anemia (SCA) is a hereditary anemia caused by homozygosity for the c.20A>T mutation in the beta-globin gene (HBB) that gives rise to hemoglobin S (Hbs). In low-oxygenated blood, Hbs molecules form polymers which stretch and deform the red blood cells and dramatically alter their mechanical and rheological properties, leading to recurrent episodes of vaso-occlusion and chronic hemolysis.

Cerebral vasculopathy is one of the most devastating complications of this disease and even young children with SCA have a high risk of stroke. It occurs in about 11% of these children before the age of 20 (1, 2). Pathophysiology of stroke is complex and the underlying mechanisms remain largely unknown (3).

Some environmental and genetic determinants are able to modulate the onset, course and outcome of the SCA. Among those, the level of fetal hemoglobin (Hbf, a2y2) has been proposed as the most significant disease modulator (4, 5). Hbf is the major form of Hb expression during gestation. Around birth, fetal to adult hemoglobin switching occurs gradually (Fig. 1a) being regulated by transcription factors, including BCL11A, MYB, and KLF1 (4, 10).

In this work, we aimed to investigate if the level of Hbf in SCA children is related with their risk of stroke and if their level of Hbf is modulated by variants in genes, such as HBG2, BCL11A, HBS1L-MYB, and KLF1.

**Materials and Methods**

Seventy-six children (9-18 years of age) with SCA were categorised according to their degree of fetal globin expression evaluated by transcriparial Doppler velocities and magnetic resonance imaging:

- **Stroke group.** Included 32 SCA children with high transcriparial Doppler (TCD) velocities, either “conditional” (170 – 199 cm/s) or “high risk” (>200 cm/s), and children with silent infarcts or cerebral vasculopathy on magnetic resonance imaging (MRI).
- **Control group.** Included 20 SCA children without previous history of stroke, normal TCD velocities and no abnormalities on MRI.

- **Hematological and imaging data** were retrospectively obtained from patients’ medical records at Greater Lisbon area hospitals.

- **Genotypic analysis** of six known SNPs was performed using PCR-RFLP or Sanger sequencing: HBG2: rs7482144; MYB: rs4895441, rs4991517 and rs3589368; BCL11A: rs13886868; and rs4671393. KLF1 gene and its promoter region (total of 3.2 kb) were analysed using Next-Generation Sequencing – Nextera XT methodology, in a MiSeq equipment, Illumina. Data analyses were performed using MiSeq reporter v2.6.2 and Integrative Genomics Viewer v2.3.86.

- **Statistical analysis** was performed with SPSS v23. Median and 95% CI were used to describe hematological parameters of patient groups. Differences in the median values of Hbf was analysed by the Mann-Whitney or Kruskal-Wallis non-parametric tests. A p value of 0.05 was considered statistical significant.

- **In silico studies** of the novel variant in KLF2 were done using Polymorph-2.

**Results and Discussion**

**I - Fetal hemoglobin level and risk of stroke in SCA**

All the sixty-seven children analysed presented homozygosity for the c.20A>T mutation in the Hbf gene, confirming they are SCA patients.

However, these SCA children presented a variable level of Hbf (median 9.3%; min 1.5; max 27.8). When they were grouped according to their degree of cerebral vasculopathy (Fig. 2), it was observed that lower Hbf levels are associated with stroke events (p<0.005).

**II - Genetic modulation of fetal hemoglobin phenotypic expression**

II.1 – SNPs in genes, such as HBG2, BCL11A and HBS1L-MYB, may modulate the phenotypic expression of Hbf in SCA. For example (Fig. 3), we have observed that SCA patients with the rarest genotypes in HBG2 (rs7482144, TT-TC) presented higher levels of Hbf (p<0.031).

Additionally, the rs13886868_C and the rs4671393_A alleles in BCL11A also seemed to predispose to higher Hbf levels.

**Fig. 1.** Fetal to adult hemoglobin switching. A) Timing of S-like globin switch during human ontogeny. The embryonic, fetal and adult stages are shown in blue, green and red, respectively. B) Regulators of hemoglobin switching, including KLF1, BCL11A, and MYB. Gene activation is depicted with an arrow, and gene repression with a blunt-ended arrow. BCL11A binding sites are indicated with an asterisk (*). C) LD-Linkage control region. HfS/Disease hypervariable sites.

**Fig. 2.** Box plots showing Hbf levels presented by SCA patients according to their degree of cerebral vasculopathy (Control; Risk; Stroke).

**Fig. 3.** Box plots showing Hbf levels presented by SCA patients according to their genotypes at HBG2, rs7482144.

**Fig. 4.** a. Schematic representation of KLF2 gene with the identification of the eleven distinct variants found: 18, 19 and 21 indicate exons 1, 2 and 3, respectively; F1, F2 and F3 depict the three zinc finger domains. b. DNA sequencing profile of the novel variant c.1030G>C found in heterozygosity in one patient. a. b. is allele analysis of the novel variant using the Polymorph-2 software.

**Conclusion**

Our results corroborate previous studies suggesting that a low level of Hbf in SCA patients is a risk factor for stroke (6).

The results from this study confirm that KLF1 is an essential modulator of Hbf expression in SCA. We report for the first time the importance of KLF1 variants in combination with other genetic modifiers (namely HBG2, BCL11A and HBS1L-MYB) to the final phenotypic expression of Hbf in SCA children with different cerebral vasculopathy. Consequently, this study allowed the delineation of a genetic pattern with prognostic value for SCA.