FETAL HEMOGLOBIN LEVEL AND STROKE RISK IN CHILDREN WITH SICKLE CELL ANEMIA

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INTRODUCTION and OBJECTIVES

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; α2γ2) 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 (Fig. 1b).

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***.

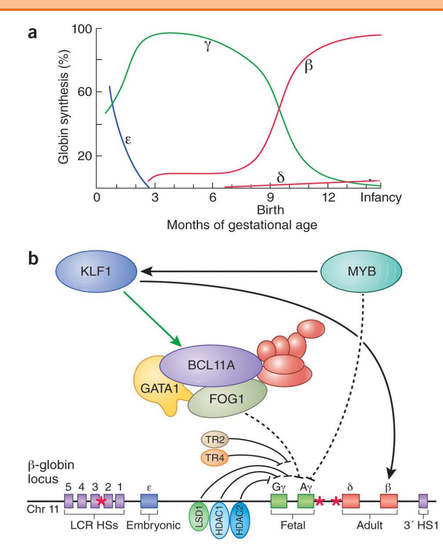


Fig. 1. Fetal to adult hemoglobin switching.

a. Timing of β-like globin subunit switching 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 (*). LCR=Locus control region; HSs= DNase hypersensitive sites.

Adapted from Sankaran and Weiss, 2015 [5]

MATERIALS and METHODS

• **Subjects**

Sixty-seven children (≥3 years of age) with SCA were categorised according to their degree of cerebral vasculopathy evaluated by transcranial Doppler velocities and magnetic resonance imaging: **Stroke group**, included 15 SCA children with history of at least one stroke episode; **Risk group**, included 32 SCA children with high transcranial 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.

• **Genotyping** of six known SNPs was performed using PCR-RFLP or Sanger sequencing: *HGB2*: rs7482144; *HBS1L-MYB:* rs4895441, rs9399137 and rs9389268; and *BCL11A*: rs11886868 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.

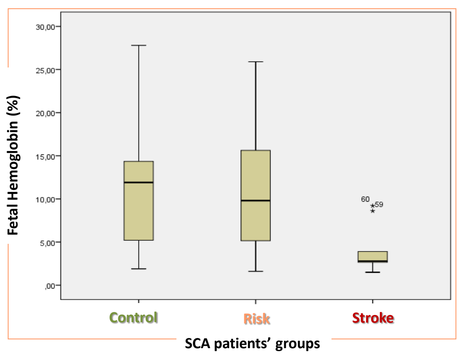
• **Statistical analyses** were performed with SPSS v23. Median and 95% CI were used to describe hematological parameters of patients in each group. Differences in the median values of HbF was analysed by the Mann-Whitney or Kruskal-Wallis non-parametric tests. A *p* value of < 0.05 was considered statistical significant.

• ***In silico* studies** of the novel variant in *KLF1* were done using *PolyPhen-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 *HBB* 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).

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

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 rs11886868_C and the rs4671393_A alleles in *BCL11A* also seemed to predispose to higher HbF levels.

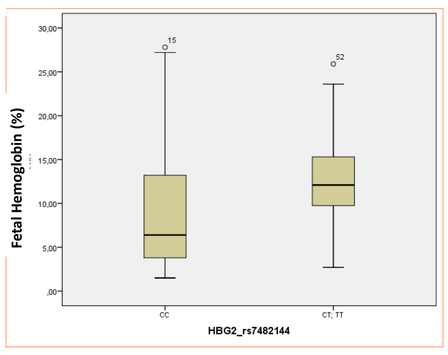


Fig. 3. Box plots showing HbF levels presented by SCA patients according to their genotype at *HBG2*, rs7482144.

II.2 – The spectrum of variants in *KLF1* gene

Eleven distinct variants were identified by NGS and validated by Sanger sequencing (Fig. 4a and b) in the analysed SCA children. We have found one novel missense variant, c.1026G>C, located at exon 3. However, *in silico* studies have predicted a benign consequence at protein level [p.Q342H]: HumDiv score = 0.013; HumVar score = 0.063 (Fig. 4c).

The majority of these SCA patients (83%) presented at least one variant in *KLF1* gene.

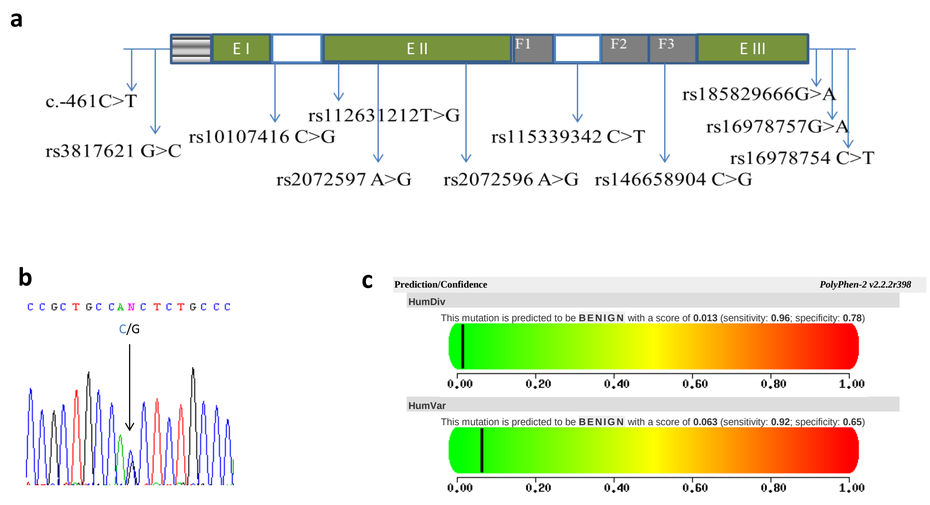


Fig. 4a. Schematic illustration of *KLF1* gene with the identification of the eleven distint variants found. EI, EII and EIII indicate exons 1, 2 and 3, respectively. F1, F2 and F3 depict the three zinc finger domains.
b. DNA sequencing profile of the novel variant c.1026G>C found in heterozygosity in one patient.
c. *In silico* analysis of the novel variant using the *Polyphen-2 sofware.*

II.3 - We further examined **the effect of the co-inheritance of *KLF1* variants and the other SNPs in *HBG2, BCL11A* and *HBS1L-MYB*.** These include the T alele at *HBG2* rs7482144, the A allele at *BCL11A* rs46671393, the G allele at *HBS1L-MYB* rs4895441 and the C allele at HBS1L-MYB rs9399137.

The wide range of HbF expression observed among the SCA patients pointed to a combined effect of *KLF1* variants and the other genetic modifier factors. In Fig. 5 is demonstrated the additive effect of these genetic modifiers for higher expression of HbF in SCA (*p*=0.021).

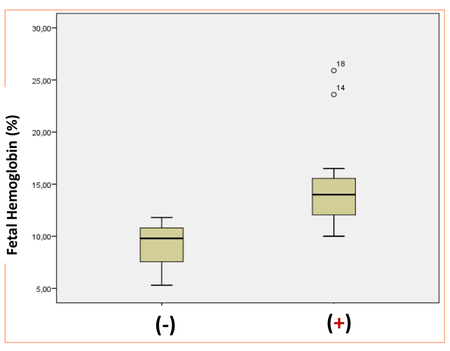


Fig. 5. Box plots showing the HbF levels presented by SCA patients **(-)** without variants in *KLF1*, *HBG2*, *MYB* and *BCL11A;* and **(+) presenting at least one variant in *KLF1* and the set of *HBG2* rs7482144_T allele, *BCL11A* rs46671393_A allele, *HBS1L-MYB* rs4895441_G allele and *HBS1L-MYB* rs9399137_C allele**.

CONCLUSIONS

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 degrees of cerebral vasculopathy. Consequently, this study allowed the delineation of a genetic pattern with prognostic value for SCA.

References

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