

1 **HbF-promoting polymorphisms may specifically reduce the residual risk of**
2 **cerebral vasculopathy in SCA children with alpha-thalassemia**

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4 **Key words:** SCA, HbF quantitative trait loci, *Xmnl*, *BCL11A*, *HBS1L-MYB*, cerebral
5 vasculopathy, alpha-globin genotype.

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9 **Conflict of interest:** none

10
11 **Abbreviations:**

12 SCA: Sickle cell anemia

13 Hb: hemoglobin

14 HbF QTL: HbF quantitative trait loci

15 VOC: vaso-occlusive crisis

16 HMIP: HBS1L-MYB intergenic region

17 AF: allelic frequency

18 SNP: single nucleotide polymorphism

19
20 **Abstract (115 words)**

21 Sickle cell anemia (SCA) is a disease characterized by abnormal red blood cell rheology.
22 Because of their effects on HbS polymerization and red blood cell deformability, alpha-
23 thalassemia and the residual HbF level are known genetic modifiers of the disease. The aim
24 of our study was to determine if the number of HbF quantitative trait loci (QTL) would also
25 favor a specific sub-phenotype of SCA as it is the case for alpha-thalassemia. Our results
26 confirmed that alpha-thalassemia protected from cerebral vasculopathy but increased the
27 risk for frequent painful vaso-occlusive crises. We also showed that more HbF-QTL may
28 provide an additional and specific protection against cerebral vasculopathy but only for
29 children with alpha-thalassemia ($-\alpha/\alpha\alpha$ or $-\alpha/-\alpha$ genotypes).

30 **Introduction**

31 Sickle cell anemia (SCA) is a severe monogenic hemoglobinopathy characterized by
32 the production of an abnormal hemoglobin S (HbS). When deoxygenated, HbS polymerizes
33 and causes the sickling of red blood cells (RBCs). Sickle RBCs are more fragile and rigid than
34 healthy RBCs, leading to chronic hemolytic anemia and frequent vaso-occlusive crisis (VOC),
35 respectively. However, the clinical expression of SCA is highly variable from one patient to
36 another and the identification of genetic modulators could be helpful in anticipating
37 appropriate clinical management (1).

38 Hemoglobin F (HbF) plays a major role in the reduction of SCA mortality and
39 morbidity (2-4). The residual HbF level is genetically determined and three major HbF
40 quantitative trait loci (HbF QTL) have been identified on the human genome, located in the
41 promoter of *HBG2* (11p15), in the *HBS1L-MYB* (HMIP) intergenic region (6q23) and in *BCL11A*
42 (2p16) introns (5). The Thalassemia Severity Score (TSS), which considers sex, alpha/beta-
43 globin genotypes and four HbF inducer polymorphisms, has been shown to positively
44 correlate with the age of the first transfusion in homozygous beta-thalassemia patients (6).
45 In SCA, patients with increased HbF level have less severe anemia and increased RBC
46 deformability (7, 8). However, it is not known whether the number of HbF QTL could be
47 specifically associated with a particular SCD clinical presentation as it is the case for alpha-
48 thalassemia which favors the viscous vaso-occlusive sub-phenotype but prevents from the
49 hemolytic one (9, 10). This study aimed to determine whether the number of HbF QTL, in
50 association or not with alpha-thalassemia, could allow predicting the clinical sub-phenotype
51 of SCA, as does the TSS in homozygous beta-thalassemia.

52

53 **Materiel and methods**

54 A cohort of 314 unrelated HbSS children with SCA (median 9.6 years; F/M sex ratio
55 167/147) regularly followed in 4 French SCA Reference centers (Lyon n = 79, Marseille n = 9,
56 Creteil n = 191 and Mayotte n = 35) was constituted. These children had been selected by
57 their local specialized physician so that they could undoubtedly fall into one of the following
58 clinical categories: **(i) Vaso-occlusive crisis group (VOC ; n = 127)** included children that were
59 put on hydroxyurea (HU) therapy for at least 3 hospitalized VOC a year or 2 ACS since birth,
60 without any sign of cerebral vasculopathy; **(ii) Vasculopathy group (VASC ; n = 112)** included
61 children attending a regular exchange transfusion program for stroke or confirmed abnormal
62 transcranial Doppler exam (time-averaged mean of maximum velocities, TAMMX, ≥ 2.0 m.s-1
63 for the middle, internal, carotid or anterior cerebral arteries) without previous history of HU
64 medication for frequent VOC; **(iii) Anemia group (ANEMIA ; n = 29)** included children with a
65 steady-state Hb level below 70 g/L without cerebral vasculopathy or frequent VOC and **(iv)**
66 **Complication-free group (FREE ; n = 46)** included children who never received chronic
67 therapy (transfusion or HU) for SCA complication and whose steady-state Hb level was 80
68 g/L or higher. All children received the same long-term clinical care including an annual
69 transcranial Doppler exam since the age of 4 years. Those whose clinical phenotype was not
70 as clear-cut (conditional TCD velocity, for example) were not enrolled.

71 All patients or legal caregivers gave their consent for genetic analysis. Alpha-
72 thalassemia status was characterized by multiplex gap-PCR to detect the five common alpha-
73 thalassemia deletions (11). The allelic frequency (AF) of alpha-thalassemia was compared
74 between groups with a Chi square test. For a subset of 158 children, a previously described
75 (2, 12) HbF-QTL score ranging from 0 to 6 and corresponding to the number of HbF-

76 promoting alleles was calculated. A representative tag-SNP for each loci was employed,
77 namely rs7482144 C>T (*XmnI*) for the β -globin gene cluster (chromosome 11), rs1427407
78 G>T for *BCL11A* (chromosome 2) and rs28384513 A>C for HMIP (chromosome 6). These SNPs
79 were genotyped using a commercial reverse dot-blot kit (Beta-Thal Modifier StripAssay®,
80 Vienna Lab, Vienna, Austria), Sanger direct sequencing or dedicated real-time PCR methods
81 (12, 13). For statistical analyses, two HbF-QTL groups were considered: patients with a score
82 of 0 or 1 versus others.

83 Chi-square and ANOVA/LSD post-hoc tests were used to compare sex and age
84 repartition between the four clinical groups. For each group, the distribution of patients in
85 the two HbF-QTL categories was compared with the distribution in the whole cohort using
86 Fisher exact test. The proportions of patients with high HbF-QTL score in each clinical group
87 were also compared side-by-side using Fisher exact test. All calculations were done
88 separately in presence or absence of alpha-thalassemia, either at the heterozygous ($-\alpha/\alpha$)
89 or homozygous state ($-\alpha/-\alpha$). The Statistical Package for the Social Sciences version 20.0
90 (SPSS Inc., Chicago, IL, USA) was used and significance was defined as $p < 0.05$.

91

92 **Results**

93 No difference in the F/M sex ratio was observed between groups ($p = 0.47$; data not
94 shown). The mean \pm SD ages of the four groups at the time of inclusion were as follows: VOC
95 12.5 ± 4.3 years, VASC 12.3 ± 3.1 , ANEMIA 11.4 ± 4.0 and FREE 10.3 ± 3.4 . Among the 314
96 SCA children, only the -3.7 Kb alpha-thalassemia deletion was identified. The global AF was
97 0.27 (109 heterozygotes $-\alpha/\alpha$ and 29 homozygotes $-\alpha/-\alpha$) with significant differences
98 according to the clinical groups ($p < 0.001$; Chi square test). The AF of the -3.7 Kb deletion

99 was significantly higher in the VOC group (0.34; $p < 0.001$) and significantly lower in the VASC
100 group (0.17; $p < 0.001$) compared to the ANEMIA and FREE groups (0.26 and 0.28,
101 respectively).

102 The genotyping results of the HbF QTL and the corresponding scores are given in
103 table I, according to the clinical group and the alpha-thalassemia status. Global AF of *BCL11A*
104 and HMIP SNPs (0.26 and 0.30, respectively) were higher than *Xmnl* (0.08), reflecting a low
105 prevalence of Senegal and Arab-Indian β^S haplotypes. As a consequence, most of the
106 patients (110 out of 158) had a score of 0 or 1. Very interestingly, in presence of alpha-
107 thalassemia at the heterozygous or homozygous state, no patient belonging to the higher
108 HbF category fall into the VASC group ($p = 0.02$). Consequently, the proportion of patients
109 with a HbF-QTLs score ≥ 2 was significantly lower compare to the three other groups (Figure
110 1B), which was absolutely not the case for patients with normal alpha-globin genotype
111 (Figure 1A). No other unbalanced repartition was observed.

112

113 Discussion

114 The protective effect of alpha-thalassemia against SCA cerebral vasculopathy is very
115 well known. However, this protection is not absolute, as reflected by our cohort where 12
116 out of 70 patients with alpha-thalassemia belonged to the vasculopathy group. There is thus
117 a room for improvement which seems to be given by the additional presence of at least two
118 favorable HbF QTLs. This observation is not very surprising per se since we already observed
119 a differential effect of the HbF QTL according to the alpha-globin genotype for VOC
120 occurrence and for some biological markers of severity (leucocytes and CRP) in a pediatric
121 Senegalese cohort (12). In that study, we could not focus on cerebral vasculopathy due to

122 the absence of regular transcranial Doppler exam in Senegal. Moreover, the Senegalese
123 children were all HU-free and none of them attended a regular exchange-transfusion
124 program. Since it was not the case in our study, we classified children according to their
125 natural clinical evolution and not according to recent clinical and biological data which could
126 obviously be influenced by HU or regular transfusions. The reliability of our clinical
127 classification was ascertained by the fact that the well-known modulating effects of alpha-
128 thalassemia (protection from cerebral vasculopathy but higher risk of frequent VOC) clearly
129 appeared, thus confirming previous studies (14, 15).

130 The protecting effects of alpha-thalassemia on cerebral vasculopathy are thought to
131 result from its effects on red blood cell deformability and hemolysis (16). However, the
132 lower hemolysis in SCA patients with alpha-thalassemia causes a rise in blood viscosity,
133 which would explain the higher frequency of VOC-like events compared to patients without
134 alpha-thalassemia (14). In addition, it has been demonstrated that SCA patients with alpha-
135 thalassemia had higher red blood cell aggregation than those without, which could further
136 increase the risk for VOC (17). Given the relatively small number of children in each
137 phenotypic category, a replication study on another independent SCA cohort (ideally
138 enriched in Senegal haplotypes for a higher *Xmnl* AF) is obviously needed to support our
139 conclusion. To increase the number of children in the VASC group, it could be envisaged to
140 consider those with a conditional TCD (TAMMX between 1.7 and 2m.s⁻¹) and silent cerebral
141 infarcts (small cortical or subcortical or larger subcortical) at magnetic resonance imaging
142 (MRI) testing. In the same way, a replication cohort enriched in patients with the β^S Senegal
143 haplotype (*Xmnl* positive) may allow to obtain patients with an HbF QTL-score of 5 or 6.

144 Friedrisch *et al.* already found that BCL11A SNPs were associated with higher HbF

145 induction under HU medication (18). Our work shows another potential use of the HbF QTL
146 for the clinical management of patients with SCA. A few different HbF genetic combined
147 variables have been described in the literature and they will undoubtedly add significant
148 value in the next future for both clinical studies and patient stratification in therapeutic
149 purpose. They all rely on the same three genetic loci (*Xmnl*, *BCL11A* and HMIP) but they vary
150 each other by the chosen tag-SNP and/or its relative weight in the model. In our HbF QTL-
151 score, there is one SNP per loci and all three loci have equal ponderation. Conversely, in the
152 4-variant model recently described by Gardner *et al.* (19), the highest ponderation is given to
153 the *BCL11A* locus for which two SNP are genotyped (rs1427407 and rs6545816). A different
154 SNP is also employed for HMIP (rs66650371 instead of rs28384513 in our study) despite its
155 very low AF in African populations without significant European admixture (2). It would be
156 very interesting to test whether a higher Gardner score is also associated with a lower
157 prevalence of cerebral vasculopathy in SCA children with alpha-thalassemia. From a
158 technical point of view, the Beta Thal Modifier Strip Assay kit represents a very simple,
159 reliable and time-efficient alternative for the genotyping of the main HbF QTLs (but not
160 *BCL11A* rs6545816) compared to the use of dedicated Sanger / real-time PCR methods.
161 Next-Generation Sequencing would be another possible alternative but care should be taken
162 for *Xmnl* which is located in a region of high homology between the *HBG2* and *HBG1* gene
163 promoters.

164 In conclusion, our work suggests a specific protective effect of the HbF-QTL on SCA
165 cerebral vasculopathy but in presence of alpha-thalassemia only (either at the homozygous
166 or heterozygous state). Apart from a necessary confirmation on replication cohorts, this
167 result would also require to be functionally ascertained by rheological measurements.
168 Theoretically, in presence of alpha thalassemia, SCA patients with at least two HbF QTL

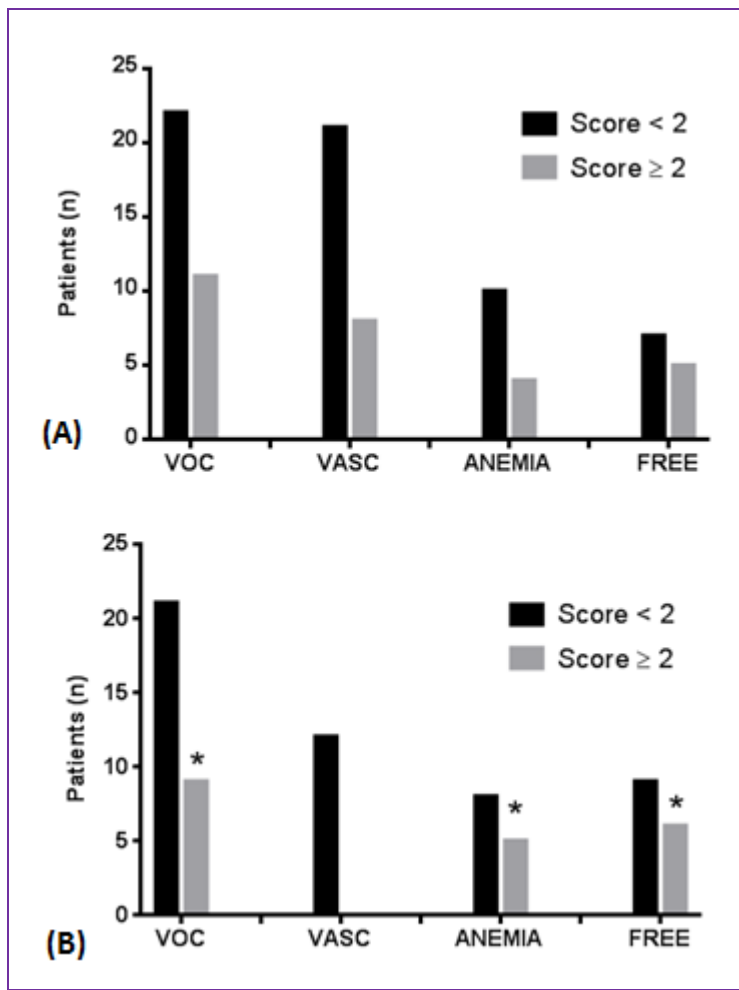
169 would be expected to have a higher deformability (but not necessarily a higher viscosity)
170 than patients with 0 or 1 HbF-QTL only. On the contrary, the number of HbF QTL would not
171 have any influence on the rheological parameters in SCA patients without alpha-thalassemia.

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233 **Figure 1:** Distribution of SCA patients with HbF QTL score < 2 or ≥ 2 according to the clinical
 234 sub-group, in absence (A) or presence (B) of alpha-thalassemia ($-\alpha/\alpha\alpha$ or $-\alpha/-\alpha$
 235 genotypes). * $p < 0.05$ at Fisher exact test versus the VASC group. No side-by-side
 236 comparison was significant.

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239 **Table I: Genotyping results of the HbF-QTLs according to the alpha-globin genotype in 158 patients with SCA**

		SCA patients with normal alpha-globin genotype ($\alpha\alpha/\alpha\alpha$) - n = 88				SCA patients with alpha-thalassemia ($-\alpha/\alpha\alpha$ or $-\alpha/-\alpha$) - n = 70			
		VOC (n = 33)	VASC (n = 29)	ANEMIA (n = 14)	FREE (n = 12)	VOC (n = 30)	VASC (n = 12)	ANEMIA (n = 13)	FREE (n = 15)
<i>Xmnl</i> (AF)		0.09	0.05	0.07	0	0.12	0	0.23	0.08
<i>BCL11A</i> rs1427407 (AF)		0.26	0.22	0.14	0.33	0.22	0.08	0.19	0.21
HMIP rs28384513 (AF)		0.24	0.33	0.32	0.33	0.25	0.13	0.15	0.46
HbF-QTL score	0 or 1 (n)	22	21	10	7	21	12	8	9
	≥ 2 (n)	11	8	4	5	9	0	5	6
	p*	0.84	0.83	1	0.52	1	0.02	0.54	0.56

240 SCA: sickle cell anemia; HbF-QTL: quantitative trait loci of hemoglobin F; AF: Allelic frequency; *: Fisher exact test versus the whole cohort.

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