

Marisa Silva¹, Sofia Vargas¹, Andreia Coelho¹, Joana Mendonça¹, Luís Vieira^{1,2}, Paula Kjollerström³, Raquel Maia³, Rita Silva⁴, Alexandra Dias⁵, Teresa Ferreira⁵, Anabela Morais⁶, Isabel Mota Soares⁷, João Lavinha^{1,8}, Paula Faustino^{1,9}

¹ Departamento de Genética Humana, Instituto Nacional de Saúde Doutor Ricardo Jorge, Lisboa; ² ToxOmics, Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Lisboa; ³ Unidade de Hematologia, Hospital de Dona Estefânia, CHLC, Lisboa; ⁴ Unidade de Neuropediatria, Hospital de Dona Estefânia, CHLC, Lisboa; ⁵ Departamento de Pediatria, Núcleo de Hematologia, Hospital Prof. Doutor Fernando da Fonseca, Amadora; ⁶ Departamento de Pediatria, Hospital de Santa Maria, CHLN, Lisboa; ⁷ Departamento de Pediatria, Hospital Garcia de Orta, Almada; ⁸ BiolSI, Faculdade de Ciências, Universidade de Lisboa, Lisboa; ⁹ ISAMB, Faculdade de Medicina, Universidade de Lisboa, Lisboa; Portugal.

1. Introduction

Sickle cell anaemia (SCA) arises from homozygosity for the mutation c.20A>T in the *HBB* gene. However, it shows a multifactorial-like behavior with high heterogeneity of clinical features¹. Cerebral vasculopathy (CVA) constitutes a severe complication of SCA, especially in children, and comprises a spectrum of clinical manifestations that includes overt stroke, transient ischemic attacks, silent cerebral infarcts (SCIs), and frequent cognitive decline^{2,3,4}. The risk of stroke development as well as of other CVA events, such as SCIs, may be modulated by underlying genetic modifiers, namely those affecting vascular homeostasis.

2. Aims

- Investigate the role of variants in **genes related with endothelial adhesion (*VCAM1* and *ITGA4*) and nitric oxide metabolism (*NOS3*)** on CVA events in SCA children;
- Study the effects of genetic variants on the levels of biochemical/haematological biomarkers of chronic haemolysis;
- Evaluate the putative additional modulating role of variants previously identified as stroke risk factors by genome-wide associated studies: *GOLGB1* Y1212C, *ENPP1* K173Q and *PON1* Q192R.

3. Methodology

- Subjects:** 70 SCA children characterized according to their CVA degree, by transcranial Doppler ultrasound [time-averaged mean of maximum velocity (TAMMV) in the middle cerebral artery], magnetic resonance imaging (MRI) or CT scan;
- Molecular analyses:** PCR, PCR-RFLP, Sanger sequencing and next-generation sequencing;
- Haplotype reconstruction:** Haploview v.2.0,
- Statistical analysis:** SPSS v.25.0.

4. Results and Discussion

Specific **genetic variants** and **haplotypes** in *VCAM1* (Table 1), *ITGA4* and *NOS3* (Table 2) were associated with **higher risk** or **protection** for the occurrence of overt ischemic stroke events or silent cerebral infarcts in children with SCA (Table 3).

Variant	<i>VCAM1</i> haplotypes						
	1	2	3	4	5	6	7
rs1409419 (T>C)	C	C	C	C	C	C	I
rs3917024 (C>T)	C	C	C	C	C	T	C
rs3917025 (CT>delCT)	CT	CT	CT	CT	--	--	CT
rs3783598 (T>G)	T	T	T	T	T	G	T
rs1041163 (T>C)	T	T	C	C	T	T	T
rs3783599 (C>T)	C	T	C	T	C	C	C

Variant	<i>NOS3</i> haplotypes						
	1	2	3	4	5	6	7
rs2070744 (C>T)	C	C	C	T	T	T	T
Intron 4 VNTR*	4a	4b	4b	4a	4b	4b	4c
rs1799983 (T>G)	G	G	T	G	G	T	G

*VNTR alleles: 4a = 4 repeats of 27bp; 4b = 5 repeats of 27bp; 4c = 6 repeats of 27bp

Table 1. (left) *VCAM1* gene promoter haplotypes.

Table 2. (above) *NOS3* haplotypes.

- VCAM1* promoter haplotype 7** as well as **rs1409419_T** alone were associated with **higher velocity of cerebral blood flow**, \uparrow TAMMV (>170 cm/s); the same was observed for ***ENPP1* K173Q (rs1044498_A)**;
- ITGA4* variants rs113276800_A** and **rs3770138_T** were associated with **stroke events**;
- NOS3* haplotype 5** as well as **VNTR 27bp_4b allele** showed a **protective effect for silent cerebral infarcts**; **haplotype 7** also showed a **protective effect for cerebral vasculopathy** globally.

Gene	Variant	Predicted Association	P	OR	CI _{95%}
<i>VCAM1</i>	rs1409419_T	\uparrow TAMMV	0.010	4.705	1.566-14.132
	haplotype 7	\uparrow TAMMV	0.010	4.705	1.566-14.132
<i>ITGA4</i>	rs113276800_A	Stroke	0.025	7.619	1.394-41.653
	rs3770138_T	Stroke	0.045	5.571	1.122-27.665
<i>NOS3</i>	VNTR 27 bp_4b allele	Silent cerebral infarcts Protective	0.041	0.114	0.016-0.808
	haplotype 5	Silent cerebral infarcts Protective	0.009	0.13	0.029-0.582
	haplotype 7	Cerebral vasculopathy Protective	0.015	0.084	0.010-0.691
<i>ENPP1</i>	rs1044498_A	\uparrow TAMMV	0.040	4.025	1.207-13.418

Table 3. *VCAM1*, *ITGA4*, *NOS3* and *ENPP1* variants with significant association to cerebral vasculopathy.

Chronic haemolysis biomarker levels also appear to be modulated by *VCAM1* and *ITGA4* variants (Table 4).

- LDH** levels were **higher** in the presence of *VCAM1* promoter **rs1409419_T** alone as well as of **haplotype 7** but **lower** in patients with *ITGA4* **haplotype 1**;
- Total **bilirubin** levels, on the other hand, were **higher** in association with *VCAM1* **rs3783613_C** allele.

Gene	Variant	Predicted Effect	p
<i>VCAM1</i>	rs1409419_T	\uparrow LDH	<0.001
	haplotype 7	\uparrow LDH	<0.001
	rs3783613_C	\uparrow Bilirubin	0.026

Gene	Variant	Predicted Effect	p
<i>ITGA4</i>	haplotype 1: rs1375493_A rs35723031_insT g. 181459459_delTT	\downarrow LDH	0.003

Table 4. *VCAM1* and *ITGA4* variants' predicted effect on chronic hemolysis biomarkers (LDH and total bilirubin).

5. Conclusion

- We have found significant association between **specific variants in genes related with endothelial adhesion (*VCAM1* and *ITGA4*) and nitric oxide metabolism (*NOS3*)** with several cerebral vasculopathy outcomes;
- Those genetic factors may be used for the **early detection** of the SCA children presenting the highest CVA risk, thus allowing an intensification of **their preventive therapeutic strategies**;
- Our findings also provide additional clues on the SCA pathophysiology and uncover features in these genes that may prove to be crucial as potential therapeutic targets.

6. References

- Kuo 2017. Genomics Insights; 10:1-10
- Switzer et al. 2006. Lancet Neurol; 5:501-512.
- Flanagan et al. 2013. Blood; 121(16):3237-3245.
- Fasano et al. 2016. Blood Cell Mol Dis; 54:17-25.

7. Contact information

- ✉ paula.faustino@insa.min-saude.pt
- ✉ marisa.silva@insa.min-saude.pt