Sickle cell anemia: chronic hemolysis and cerebral vasculopathy

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Sickle cell anemia – molecular basis

• **Gene variant**: \( HBB \) 6th codon, \( GAG \rightarrow GTG \); \( HBB:c.20A>T \)
• **Protein variant**: \( p.Glu6Val \)

The mutation originates a hemoglobin variant named **Hemoglobin S (Hb S)**, as opposed to the normal adult Hb A.

Hb S ability to polymerize when deoxygenated gives rise to abnormal sickled red blood cells.
SCA is the most common autosomal recessive hereditary anemia in Africa

Multiple origin of SCA mutation ($HBB:c.20A>T$) in Africa

Positive selection – carrier advantage, protection against malaria
Sickle cell anemia in Portugal

- Prevalence of sickle cell anemia carriers:
  - ≈ 0% - North of Portugal
  - ≈ 1.1% - South of Portugal
  - ≈ 5-6% - High prevalence pockets

  Positive selection – carrier advantage, protection against malaria

- Absence of Portuguese SCD patient registries:
  - ≈ 600 sickle cell disease patients.

Lavinha et al, Hum Biol 1992;
Martins et al, J Med Genet 1993;
Inez et al, Arq INSA 1993.
SCA – clinical manifestations

SCD is characterized by **recurrent episodes** of severe **vaso-occlusion**, **hemolysis** and **infection**.

Sickle cells are destroyed rapidly (**chronic hemolysis**) causing **anemia**, **jaundice** and the formation of **gallstones**.

The sickle cells also block the flow of blood through vessels resulting in lung tissue damage (**acute chest syndrome**), **pain episodes** (arms, legs, chest and abdomen), **stroke** and **priapism**.

It also causes damage to most organs including the **spleen**, **kidneys** and **liver**. Damage to the spleen makes SCD patients easily overwhelmed by **infections**.
SCA – heterogeneity of clinical manifestations

SCA is a monogenic disorder with a multifatorial-like behaviour and a clinical phenotype heterogeneity inter- and intra-patients.

Several genetic and environmental modifiers have been suggested to modulate the onset and course of the disease.
Intravascular hemolysis reduces Nitric Oxide (NO) bioactivity

**Cell-free plasma hemoglobin** inactivates NO, generating metahemoglobin and inert nitrate (A).

Plasma **arginase** consumes plasma L-arginine to ornithine, depleting its availability for NO production (B).

NO is also consumed by reactions with **reactive oxygen species** ($O_2$) producing oxygen radicals like peroxynitrite (ONOO-) (C).
I - Study of the genetic modulators of intravascular hemolysis in SCA

Objective

- To study association between intravascular hemolysis level using commonly measured hemolysis biomarkers (biochemical and hematological parameters) and the inheritance of genetic variants of several candidate genes.
Genetic modulators of hemolysis in SCA

Patients clinical data at steady-state have been captured to a database in a longitudinally observed series of paediatric SCA patients.

<table>
<thead>
<tr>
<th>SCA evaluated patients</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No. of patients</strong> (ethnicity)</td>
<td><strong>99</strong> (61% Angolan; 97% of Sub-Saharan origin ancestry)</td>
</tr>
<tr>
<td>Male/Female ratio</td>
<td>1.17</td>
</tr>
<tr>
<td><strong>Current age (year)</strong></td>
<td></td>
</tr>
<tr>
<td>median</td>
<td>9.9</td>
</tr>
<tr>
<td>interquartile range</td>
<td>6.7-12.6</td>
</tr>
<tr>
<td>total range</td>
<td>2.9 – 21.7</td>
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<tr>
<td><strong>Entry age (year)</strong></td>
<td></td>
</tr>
<tr>
<td>median</td>
<td>2.3</td>
</tr>
<tr>
<td>interquartile range</td>
<td>0.7-4.7</td>
</tr>
<tr>
<td>total range</td>
<td>0.1 – 16.9</td>
</tr>
<tr>
<td><strong>Total follow-up</strong> (person*year)</td>
<td>557</td>
</tr>
<tr>
<td><strong>Follow-up/patient</strong> (median; year)</td>
<td>5.0</td>
</tr>
</tbody>
</table>
Genetic modulators of hemolysis in SCA

**Hemolysis biomarkers:**
Serum LDH, total bilirubin and reticulocyte count.

**Candidate gene genotyping:**
41 genetic variants within 13 candidate genes
- related with fetal hemoglobin level: BCL11A, HBS1L-MYB; HBB cluster (including HBG)
- alpha-thalassaemia (HBA)
- red blood cell vascular adhesion (VCAM 1, THBS 1, CD36, EDN1, ITGA4)
- and vascular tonus: (NOS3, HMOX-1)
- inflammation (TNF α)

**Statistical analysis:**
Association studies were performed using T test ANOVA parametric tests (LDH, total bilirubin) or Mann-Whitney/Kuskal-Wallis non-parametric tests (reticulocyte count), all performed with SPSS v20.0 software.
A correction for multiple testing (false discovery rate) was done.
Genetic modulators of hemolysis in SCA

Results

Hematological and biochemical markers of hemolysis vs Genetic variants
## Genetic modulators of hemolysis in SCA

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP reference</th>
<th>Allele&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Associated Allele or Haplotype</th>
<th>Presence of associated allele or haplotype</th>
<th>No. of patients</th>
<th>Haemolysis biomarkers&lt;sup&gt;b&lt;/sup&gt;</th>
<th>p-value</th>
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<tbody>
<tr>
<td>VCAM1-gene</td>
<td>rs3783613</td>
<td>G/C</td>
<td>C</td>
<td>Yes</td>
<td>12</td>
<td>LDH (U/L) mean±SD 1270.6±279.3</td>
<td>p=0.002</td>
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<td></td>
<td>rs3176878</td>
<td>C/T</td>
<td>C</td>
<td>No</td>
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<td>LDH (U/L) mean±SD 926.6±348.4</td>
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<td></td>
<td>rs3783615</td>
<td>A/T</td>
<td>A</td>
<td>VCAM1_g_hapl 7</td>
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<td>A/G</td>
<td>A</td>
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<tr>
<td>VCAM1-promoter</td>
<td>rs1409419</td>
<td>C/T</td>
<td>C</td>
<td>VCAM1_p_hapl 9</td>
<td>4</td>
<td>LDH (U/L) mean±SD 1.65±0.05</td>
<td>p&lt;0.001</td>
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<tr>
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<td>rs3917024</td>
<td>C/T</td>
<td>T</td>
<td>VCAM1_p_hapl 9</td>
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<td>T</td>
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<td>C/T</td>
<td>T</td>
<td>VCAM1_p_hapl 9</td>
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<td>CD36</td>
<td>rs1984112</td>
<td>A/G</td>
<td>G</td>
<td>VCAM1_p_hapl 9</td>
<td></td>
<td>Reticulocyte count (%) 13.06±0.80</td>
<td>p=0.001</td>
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<tr>
<td></td>
<td>5'UTR</td>
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<td>NOS3</td>
<td>rs2070744</td>
<td>C/T</td>
<td>T</td>
<td>VCAM1_p_hapl 9</td>
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<td>Reticulocyte count (%) 2.41±0.42</td>
<td>p&lt;0.001</td>
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<tr>
<td></td>
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<td></td>
<td>2</td>
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<tr>
<td>HBA</td>
<td>del 3.7kb</td>
<td>Non-del/del</td>
<td>del</td>
<td>VCAM1_p_hapl 9</td>
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<td>Reticulocyte count (%) 2.04±0.40</td>
<td>p=0.002</td>
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<td>53</td>
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</tr>
</tbody>
</table>

<sup>a</sup> Reference allele.

<sup>b</sup> Data presented as mean±SD.

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Coelho A <i>et al</i>. Eur J Haematol, 2014
A diminished reticulocyte count was associated to the 3.7kb deletion at *HBA*, whereas an elevated count was associated to rs1984112_G allele at *CD36*.
Toward a hemolytic genetic profile in sickle cell anemia

<table>
<thead>
<tr>
<th></th>
<th>CD36 (rs1984112)_G allele</th>
<th>Genetic profile identification</th>
<th>No. of SCA patients</th>
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</thead>
<tbody>
<tr>
<td>absence</td>
<td>presence</td>
<td>2:1</td>
<td>27</td>
</tr>
<tr>
<td>absence</td>
<td>absence</td>
<td>2:2</td>
<td>25</td>
</tr>
<tr>
<td>presence</td>
<td>presence</td>
<td>1:1</td>
<td>17</td>
</tr>
<tr>
<td>presence</td>
<td>absence</td>
<td>1:2</td>
<td>25</td>
</tr>
</tbody>
</table>

Categorisation of SCA patients into genetic profiles concerning theirs HBA and CD36 variants, **assuming a dominant model of inheritance.**
Toward a hemolytic genetic profile in sickle cell anemia

Our data are compatible with a synergetic interaction between $HBA_{3.7 \text{ del}}$ and $CD36\ (rs1984112)\_G$ for the assumed model of inheritance.

Steady-state reticulocyte count as a function of the $HBA$-$CD36$ profile (median; interquartile range; minimum and maximum values).

$P$-values marked above each comparison (Mann-Whitney test)

Dashed horizontal line, reticulocyte count in the general population.

Our data are compatible with a synergetic interaction between $HBA\_3.7\ \text{del}$ and $CD36\ (rs1984112)\_G$ for the assumed model of inheritance.
Hemolysis in SCA - genetic modifiers

1. Alpha-thalassaemia

The presence of the 3.7 kb deletion alpha-thalassaemia determinant at the HBA gene were found associated with low levels of haemolysis, measured by low levels of total bilirubin and reticulocyte count. SCA patients who co-inherited the deletion have reduced haemolysis owing to a lower intracellular concentration of HbS that in turn decreases HbS polymer-induced cellular damage.

- Alpha-thalassaemia was one of the first identified genetic modifier of SCD
- Due to the African ancestry of the SCA patients in this series, a high frequency (0.22) of the 3.7kb alpha-thalassaemia deletion was found.
Hemolysis in SCD - genetic modifiers

2. Cell vascular adhesion

Vascular adhesion molecule-1 (VCAM-1) and Cluster of differentiation 36 (CD36)
Vascular adhesion molecule-1 (VCAM-1), is a glycoprotein present at surface of endothelial cells following cytokine stimulation that mediates the adhesion of monocytes, lymphocytes and neutrophils to the endothelium of both large and small blood vessels.
Stress reticulocytes and sickle erythrocytes have a propensity to adhere to VCAM-1 via the very late antigen-4 (VLA-4) or integrin (α4β1) expressed on their surface membrane.

Hypoxia induces Integrin (α4β1)/VCAM-1 interaction to increase adhesion.
Adhesion molecule: CD36

Cluster of differentiation 36 (CD36) is expressed in many type of cells including microvascular endothelium cells, monocytes/macrophages, platelets, microglia, and erythroid precursors.

CD36 molecules are present at higher levels on stress reticulocytes and sickle erythrocytes.

CD36 is a common mediator for neurological and vascular diseases

Hypertension, Dyslipidemia and Diabetes present in common elevated expression of CD36 and higher incidence of vascular diseases.

Adhesion molecule: CD36

Several CD36 pathways activated by distinct ligands elicit inflammatory responses.

The binding of thrombospondins (TSPs) to CD 36 produces pro-inflammatory factors and causes vascular dysfunction and endothelial apoptosis.

Thrombospondin are extracellular matrix proteins that mediate cell-cell interaction. They promote greater adhesion of sickle RBCs to the microvascular endothelium.
Adhesion molecules - results

- **VCAM1_gene_haplotype 7** was found associated with **higher levels of LDH**, suggesting a relation between this variant and a sub-phenotype characterised by **more severe haemolysis (risk factor)**. Contrarily, heterozygosity for **VCAM1_promotor_haplotype 9** was found associated with **lower levels of total bilirubin** revealing a **protective effect against hemolysis**.

- The **rs1984112_G** allele located in the 5’UTR of the **CD36** gene revealed to be associated with higher levels of reticulocyte count. – **risk factor** of SCA hemolysis

- The rs1984112_G allele was already reported as a modulator of cholesterolemia and tissue vitamin E uptake.

**Aims:**

**In silico** studies – Transcriptional binding factor analyses

Functional studies

Underlying mechanisms
Adhesion molecules

Target adhesion molecules to promote endothelial function?

In mice, genetic ablation of CD36 is associated with a less inflammatory state and delays in vascular disease progression (Qin, J Neurosci. 2011)

Several pharmacological agents have been identified to reduce CD36 expression and functions (reviewed in Sunghee, Curr Pharm Des, 2012)

CD36 antagonists:
- Small-molecules based on a CD36-binding peptide sequence from TSP1 are being tested.
- Statins – they suppress oxLDL uptake and down-regulate CD36 expression
Endothelial Nitric Oxide Synthase (eNOS), also known as nitric oxide synthase 3 (NOS3) is an enzyme encoded by the NOS3 gene.

NOS3 in endothelial cells generates nitric oxide (NO), a gas with potent vasodilation and antiadhesive properties. NO is a critical molecule for proper endothelial function and maintain of a patent vascular lumen.

NO also plays a role in cellular proliferation, leukocyte adhesion, and platelet aggregation. It also presents antioxidative activities, such us superoxide scavenging and heme oxygenase induction.
In our study, the rs2070744_\text{T} allele at NOS3 promoter seems to have a protective effect on SCA haemolysis as it was found associated with lower bilirubin levels.

On the other hand, the rs2070744_\text{C} allele has already been considerate a genetic risk factor of coronary heart disease and rheumatoid arthritis. (Cattaruzza et al, Circ Res 2004; Melchers et al, Arthritis Rheum 2006).

NOS3 promoter rs2070744 – A modulator of NOS3 transcription?

- \textit{In silico studies} – Transcriptional binding factor analyses
- \textit{In vitro expression studies}
- Underlying mechanism

- NO donors (citrulline)
II - Hemolysis in SCD and Cerebral Vasculopathy

Objectives:

- To study association between cerebral vasculopathy and intravascular hemolysis level using commonly measured haemolysis biomarkers (biochemical and haematological parameters).

- To search for associations between putative genetic modifiers of vascular tonus, vascular cell adhesion and inflammation, and the risk for stroke, in the context of SCD in pediatric patients.
Cerebral Vasculopathy in SCD

- **Overt stroke (ischemic)** = abrupt focal neurological deficit with corresponding evidence of cerebral infarct on neuroimaging (Magnetic Resonance Imaging, MRI); locates in the cortex and deep in the white matter, with large dimension. May result in permanent physical or neuropsychological impairment.

SCD children have 221-times increased risk for the occurrence of overt stroke than other children.
A head computed tomography scan in a 2 years old Portuguese child with sickle cell anemia: extensive acute infarction of the left middle and posterior cerebral arteries; atrophy of the right brain hemisphere.

Cerebral Vasculopathy in SCD

- **Silent infarct** = area of intensified signal on cerebral MRI, without history or physical findings associated with focal deficit; deep in the white matter of (mostly) frontal and parietal lobes, with smaller size.

  Associated to **cognitive function impairment**. Most common cause of **neurological disease** in children with SCD;

  **SCD children have 410-times increased risk to develop cerebral infarcts than other children.**

Risk factor for overt stroke occurrence (14-fold)

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>Overt Stroke</th>
<th>Silent Cerebral Infarcts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>• Elevated WBC counts</td>
<td>• Seizures</td>
</tr>
<tr>
<td></td>
<td>• Low Hb</td>
<td>• Low Hb</td>
</tr>
<tr>
<td></td>
<td>• Relative hypertension</td>
<td>• Systolic hypertension (adults)</td>
</tr>
<tr>
<td></td>
<td>• Increased frequency of ACS</td>
<td>• Male gender</td>
</tr>
<tr>
<td></td>
<td>• Nocturnal hypoxemia</td>
<td>• Elevated RBC counts</td>
</tr>
<tr>
<td></td>
<td>• Presence of SCI</td>
<td>• Elevated WBC counts</td>
</tr>
</tbody>
</table>
Cerebral Vasculopathy in SCD

- **Diagnosis of the risk to develop Stroke** –

  Blood flow at the medial cerebral artery

  Transcranial Doppler (TCD) → time-averaged mean of maximum velocity (TAMMV)

    - < 170 cm/s: “normal” = average risk
    - 170 – 199 cm/s : “conditional” = moderate risk
    - > 200 cm/s = high risk

  Magnetic resonance imaging (MRI) → assessment of SCIs
**DATABASE**, containing all relevant demographic, clinical, hematological, biochemical and imaging information retrospectively collected, from hospital records of SCD children.

**POPULATION SAMPLE:**

- **66 children (4-16 years) with SCD**: 65 $\beta^S\beta^S$ + 1 $\beta^S\beta^0$-thal
- 29 females (43.9%) and 37 males (56.1%)
- All with African ancestry: Angola, Cape Verde, Guinea-Bissau, São Tomé and Príncipe and Nigeria.
- 4 Hospitals: HDE (n=25); HSM (n=23); HFF (n=17); HGO (n=1).

**GROUP CRITERIA:**

- **Stroke** (n=13) → at least one episode of stroke (5 – 13 yrs)
- **Risk** (n=29) → “conditional” or high risk (TAMMV > 170 cm/s) and/or SCI on MRI
- **Control** (n=24) → without stroke, without SCI, TCD velocities < 170 cm/s
Molecular characterisation of the sickle cell anemia mutation
(homozygosity or compound heterozygosity)

- Molecular characterisation 22 polymorphic regions
(SNPs, indels, STRs) in genes related to

  - vascular cell adhesion (**VCAM 1, THBS 1, CD36**),
  - vascular tonus (**NOS 3, EDN 1, HMOX 1**)
  - inflammation (**TNF α**)

as well as in known globin expression modulators:
  - **HBB cluster haplotype**
  - **HBA genotype**
  - **BCL11A genotype**
Data analysis.

Association studies between severity groups (stroke, risk, control) and the genetic variants and hematological and biochemical data of patients.

1. Allelic and genotypic counts and frequencies for the overall population sample
2. Checking of Hardy-Weinberg Equilibrium (USING R SOFTWARE)
3. Association studies (contingency tables for Fisher’s exact test and Odds Ratio
4. False Discovery Rate
5. Clinical/hematological/biochemical parameters analysis with Wilcox-Mann-Whitney test and boxplots visualisation
Stroke risk in SCD

Hematological and Biochemical markers

VS

Stroke risk
Lower Hb F levels associate with stroke

HbSF subphenotype $\rightarrow >10\%$ HbF

Mean HbS (66 patients) = 80.59 %
Mean HbS (High HbF – 36 patients) = 74.95 %
Mean HbS (Low HbF – 30 patients) = 87.30 %

<table>
<thead>
<tr>
<th>Hematological parameter</th>
<th>Groups</th>
<th>Wilcoxon-Mann-Whitney test for homogeneity (p-value)</th>
<th>Contingency Table</th>
<th>Association</th>
<th>OR</th>
<th>Associated group</th>
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<tbody>
<tr>
<td>Fetal hemoglobin</td>
<td>Stroke Control</td>
<td>0.008</td>
<td>Stroke</td>
<td>Fisher’s exact test</td>
<td>0.037</td>
<td>Stroke (Low HbF)</td>
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<td>Stroke Risk</td>
<td>0.002</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>0.007*</td>
<td>Control</td>
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<tr>
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<td>Stroke Control</td>
<td>0.013*</td>
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<td>Stroke Risk</td>
<td>0.002</td>
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</tbody>
</table>
Disease severity scores and HbF

Disease Severity Score (DSS)

Pediatric Severity Score (PSS)

\[ p = 0.004 \]

\[ p = 0.006 \]

DSS and PSS are both negatively associated with HbF.

\[ \text{Hb F} \downarrow \quad - \quad \text{Risk factor} \quad - \quad \text{disease severity} \quad - \quad \text{stroke} \]
Stroke risk association with **level of hemolysis**

Higher LDH levels are associated with the **Risk group** which probably means that this proximal hemolytic marker is closely related with the initial stage of cerebral vasculopathy.

Reticulocyte count difference between groups are not significant but suggest a tendency.

**Hemolysis levels↑**

**Risk factor** - stroke
Stroke risk in SCD

Genetic variants

VS

Stroke risk
## Association between candidate gene variants and stroke risk in SCD

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genetic variant</th>
<th>Associated Allele</th>
<th>Mode of transmission</th>
<th>Association with phenotypic groups</th>
<th>Contingency Table</th>
<th>Association</th>
<th>OR (95% CI)</th>
<th>Associated group</th>
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<tbody>
<tr>
<td><strong>VCAM 1 promoter</strong></td>
<td>rs1409419</td>
<td>Allele count (T)</td>
<td>Dominant (TT+CT)</td>
<td>Group</td>
<td>Presence n</td>
<td>Absence n</td>
<td>Fisher’s exact test p</td>
<td>OR (95% CI)</td>
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<td></td>
<td>g.100717840 T&gt;C</td>
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<td>Stroke</td>
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<td>(1.391 – 14.257)</td>
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<td>Control</td>
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<td>(1.407 – 97.351)</td>
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<td><strong>NOS 3</strong></td>
<td>rs2070744</td>
<td>Overdominant (TC)</td>
<td>Allele count (C)</td>
<td>Group</td>
<td>Presence n</td>
<td>Absence n</td>
<td>Fisher’s exact test p</td>
<td>OR (95% CI)</td>
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<tr>
<td></td>
<td>g.150992991 C&gt;T</td>
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<td>Stroke</td>
<td>6</td>
<td>7</td>
<td>0.013</td>
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<td>Control</td>
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<td>0.067*</td>
<td>(1.221 – 107.964)</td>
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<td>20</td>
<td>0.019</td>
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<td>VNTR 27 bp</td>
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<td>Allele count (4a)</td>
<td>Group</td>
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<td>Absence n</td>
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<td>OR (95% CI)</td>
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<tr>
<td></td>
<td>4a/4b/4c</td>
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<td>Risk</td>
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<td>Control</td>
<td>11</td>
<td>13</td>
<td>0.1218*</td>
<td>(1.178 – 18.321)</td>
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<td>Risk</td>
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<td>Control</td>
<td>11</td>
<td>37</td>
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<td>(1.088 – 7.088)</td>
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<td><strong>HMOX 1 promoter</strong></td>
<td>rs3074372</td>
<td>Dominant (L/L + other/L)</td>
<td>Allele count (L)</td>
<td>Group</td>
<td>Presence n</td>
<td>Absence n</td>
<td>Fisher’s exact test p</td>
<td>OR (95% CI)</td>
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<tr>
<td></td>
<td>(STR – GT)</td>
<td></td>
<td></td>
<td>Stroke</td>
<td>10</td>
<td>3</td>
<td>0.019</td>
<td>6.04</td>
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<td>S/M/L</td>
<td></td>
<td></td>
<td>Risk</td>
<td>10</td>
<td>19</td>
<td>0.148*</td>
<td>(1.196 – 42.056)</td>
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<td>Stroke</td>
<td>14</td>
<td>12</td>
<td>0.012</td>
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<td></td>
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<td>Risk</td>
<td>14</td>
<td>44</td>
<td>0.148*</td>
<td>(1.233 – 10.902)</td>
</tr>
</tbody>
</table>

*NOS 3 VNTR 4a=4x 27 bp; 4b=5x 27bp; HMOX 1 STR-L = (GT)n, n≥35*
**VCAM-1** promoter SNPs haplotypes

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>rs1409419</th>
<th>rs3917024</th>
<th>rs3917025</th>
<th>rs3978598</th>
<th>rs1041163</th>
<th>rs3783599</th>
<th>Frequency</th>
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<tbody>
<tr>
<td>#1</td>
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<td>C</td>
<td>CT</td>
<td>T</td>
<td>T</td>
<td>C</td>
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<td>T</td>
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<tr>
<td>#3</td>
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<td>C</td>
<td>CT</td>
<td>T</td>
<td>C</td>
<td>C</td>
<td>0.144</td>
</tr>
<tr>
<td>#4</td>
<td>C</td>
<td>C</td>
<td>CT</td>
<td>T</td>
<td>C</td>
<td>T</td>
<td>0.106</td>
</tr>
<tr>
<td>#5</td>
<td>C</td>
<td>C</td>
<td>delCT</td>
<td>T</td>
<td>T</td>
<td>C</td>
<td>0.038</td>
</tr>
<tr>
<td>#6</td>
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<td>delCT</td>
<td>G</td>
<td>T</td>
<td>C</td>
<td>0.030</td>
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<td>#7</td>
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<td>C</td>
<td>CT</td>
<td>T</td>
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### Association to phenotypic groups

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genetic variant</th>
<th>Associated Allele</th>
<th>Mode of transmission</th>
<th>Stroke Group</th>
<th>Control Group</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>VCAM1-</strong></td>
<td>rs1409419</td>
<td>T</td>
<td>Allele count (T)</td>
<td>Stroke</td>
<td>Control</td>
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<tr>
<td><strong>promoter</strong></td>
<td>g.100717840 T&gt;C</td>
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- **Risk allele** (not yet reported)

- On the other hand, rs1409419 C was found associated with lower levels of bilirubin (1st study)
### NOS3 polymorphisms haplotypes

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>rs2070744</th>
<th>VNTR 27 bp</th>
<th>rs1799983</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
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<td>G</td>
<td>0.08</td>
</tr>
<tr>
<td>#2</td>
<td>C</td>
<td>4b</td>
<td>G</td>
<td>0.02</td>
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<tr>
<td>#3</td>
<td>C</td>
<td>4b</td>
<td>T</td>
<td>0.03</td>
</tr>
<tr>
<td>#4</td>
<td>T</td>
<td>4a</td>
<td>G</td>
<td>0.27</td>
</tr>
<tr>
<td>#5</td>
<td>T</td>
<td>4b</td>
<td>G</td>
<td>0.45</td>
</tr>
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<td>#6</td>
<td>T</td>
<td>4b</td>
<td>T</td>
<td>0.05</td>
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<tr>
<td>#7</td>
<td>T</td>
<td>4c</td>
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</table>

### Gene: NOS3

<table>
<thead>
<tr>
<th>Genetic variant</th>
<th>Mode of transmission</th>
<th>Contingency Table</th>
<th>Association to phenotypic groups</th>
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</thead>
<tbody>
<tr>
<td>rs2070744 g.150992991 C&gt;T promoter</td>
<td>Overdominant (TC)</td>
<td>Group</td>
<td>Presence</td>
</tr>
<tr>
<td>C</td>
<td>Stroke Control</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Stroke Control</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>rs2070744 VNTR 27 bp 4a/4b/4c Intron 4</td>
<td>Dominant (4a + 4x4a)</td>
<td>Risk Control</td>
<td>23</td>
</tr>
<tr>
<td>4a</td>
<td>Risk Control</td>
<td>26</td>
<td>11</td>
</tr>
<tr>
<td>4b</td>
<td>Risk Control</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>5x 27bp</td>
<td>Risk Control</td>
<td>25</td>
<td>31</td>
</tr>
</tbody>
</table>

### Summary

- **Overdominant**:
  - Stroke Control: 6/2 (OR 8.75)
  - Stroke Control: 6/2 (OR 6.70)
- **Dominant**: (4a + 4x4a)
  - Risk Control: 23/11 (OR 4.89)
  - Risk Control: 26/11 (OR 2.71)
  - Risk Control: 3/8 (OR 0.24)
  - Risk Control: 25/31 (OR 0.42)

- **Haplotypes**:
  - #1: C-4a-G
  - #2: C-4b-G
  - #3: C-4b-T
  - #4: T-4a-G
  - #5: T-4b-G
  - #6: T-4b-T
  - #7: T-4c-G

- **Promoter**:
  - rs2070744
  - VNTR 27 bp 4a/4b/4c
  - Intron 4
NOS3: rs2070744 and VNTR 27 bp

Owusu-Ansah A, et al. 2015

NOS3, Haplotype #1 (rs2070744_C / VNTR 4a)  
- NOS3 expression↓  NO↓  →  **Risk Haplotype**

- The rs2070744_C allele has already been considered a genetic increased risk factor of coronary heart disease and rheumatoid arthritis ☑
- On the other hand, rs2070744_T was found associated with lower levels of bilirubin (1st study)  Protective for hemolysis ☑
**HMOX-1: rs3074372**

**Gene** | **Genetic variant** | **Associated allele** | **Mode of transmission** | **Association to phenotypic groups** |
---|---|---|---|---|
| HMOX-1 | rs3074372 (STR: duplGT) | Dominant (L/L + other/L) | Stroke | Fisher’s exact test |
| | | Allele count (L) | Risk | OR (95% CI) |

- | | | Presence | Absence | 0.019 | 6.04 (1.196 – 42.056) |
- | | | Stroke | 10 | 3 | 0.148* |
- | | | Risk | 10 | 19 | 3.60 (1.233 – 10.902) |
- | | | Stroke | 14 | 12 | 0.148* |
- | | | Risk | 14 | 44 |  | 

Stroke

rs3074372 is a highly polymorphic (GT)n microsatellite in gene promoter; Long allele n≥35

**Longer STR**

**Decreased HMOX-1 transcription**

**Lower levels of HO-1**

Free heme is not adequately removed and further scavenges NO molecules.

→ **Functional studies should be done.**

Adapted from Stocker and Perrella, 2006
Strokes in children with SCD

progressive stenosis of arteries due to intimal proliferation

1. Abnormal adherence
2. High rate hemolysis
3. Leukocyte aggregation
4. Vaso-constriction
5. Intimal proliferation
6. Platelet aggregation
7. Vasculopathy
8. Occlusion

Cerebral Vasculopathy in SCD

Adapted from Switzer et al., 2006.
This study contributed to the line of evidence that stroke is a consequence of the hemolysis rate – endothelial dysfunction in SCD.

This was the first study to evidence a protective role of HbF in stroke occurrence.

Beginning to delineate a profile of genetic biomarkers able to predict the risk of stroke occurrence in SCD pediatric patients. It will include:

- genetic variants related with an increased synthesis of vascular cell adhesion molecules (i.e., rs1409419_allele T of VCAM1 gene promoter)
- genetic variants related with a decreased rate of transcription of NOS3 (i.e., rs2070744_C / VNTR 4a) and HMOX1 (rs3074372_allele L) that give rise to lower levels of NO and heme oxygenase.
Future perspectives:

• To **validate** the results in a larger patient cohort with a longer follow-up.

• A number of mechanistic hypotheses compatible with the observed genotype/phenotype were proposed but **further mechanistic studies** are needed to a better understanding of the inter- and intra-individual clinical variability.

• **Functional studies** are crucial in understanding the role of genetic variants in **disease pathophysiology and evaluate potential therapy targets**.
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