UNIVERSIDADE DE LISBOA

INSTITUTO SUPERIOR TÉCNICO

GENETIC MODULATION OF PEDIATRIC CEREBRAL VASCULOPATHY IN SICKLE CELL ANEMIA

Sandra Marisa Duarte da Silva

Supervisor: Doctor Maria Paula Duarte Faustino Gonçalves

Co-Supervisor: Doctor Arsénio do Carmo Sales Mendes Fialho

Thesis approved in public session to obtain the PhD Degree in Biotechnology and Biosciences

Jury final classification: Pass with Distinction

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2021

Genetic Modulation of Pediatric Cerebral Vasculopathy in Sickle Cell Anemia

The research presented in this thesis was carried out at the *Grupo de Investigação em Hemoglobinopatias, Metabolismo do Ferro e Patologias Associadas*, *Departamento de Genética Humana*, *Instituto Nacional de Saúde Dr. Ricardo Jorge* (INSA), Lisboa, Portugal. The group's Principal Investigator, Doctor Paula Faustino (INSA) supervised the work and Doctor Arsénio Fialho (*Instituto Superior Técnico*, IST) co-supervised, under a doctoral training cooperation protocol, between INSA and IST.

For reasons of consistency, some terms were standardized throughout the thesis text. Consequently, there may be some differences in this respect between the chapters presented and the articles on which they are based.

Para os meus pais.

Das fraquezas, forças. Da dor, resiliência.

If you can't fly, then run
If you can't run, then walk.
If you can't walk, then crawl,
but by all means, keep moving.
M.L. King Jr

Sometimes it is the people no one imagines anything of
who do the things no one can imagine.
A. Turing

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RESUMO

A drepanocitose é uma patologia genética resultante da homozigotia para a mutação *HBB*:c.20A>T, a qual origina a hemoglobina S. Esta, em condições de hipoxia, polimeriza no interior dos eritrócitos conferindo-lhes uma morfologia falciforme, aumento da rigidez e fragilidade. Consequentemente, desencadeiam-se mecanismos fisiopatológicos complexos, incluindo inflamação, adesão celular, stresse oxidativo e vaso-oclusão. A doença é fenotipicamente heterogénea devido à ação moduladora de fatores ambientais e genéticos. Uma das manifestações com maior impacto é a vasculopatia cerebral pediátrica, nomeadamente o AVC isquémico e o enfarte cerebral silencioso.

Neste trabalho pretendeu-se identificar o potencial modulador de variantes em genes relacionados, por exemplo, com a adesão de células sanguíneas ao endotélio (*VCAM1, ITGA4)* ou com o tónus vascular (*NOS3*), no risco e ocorrência da referida vasculopatia cerebral.

Efetuámos estudos de associação genótipo/fenótipo em 70 crianças com drepanocitose, previamente caracterizadas em termos fenotípicos. Seguidamente, realizámos análises *in silico* para as variantes genéticas potencialmente moduladoras dos fenótipos e, por fim, estudos funcionais com modelos de células endoteliais de origem macrovascular e microvascular cerebral.

Verificámos que existe uma associação positiva entre a presença de variantes no promotor de *VCAM1* (haplotipo 7 e rs1409419_T), assim como em *ITGA4* (rs113276800_A e rs3770138_T), e a ocorrência de AVC. As análises *in silico* indicaram a potencial alteração de locais de ligação de fatores transcricionais devido à presença da rs1409419_T. Por outro lado, os estudos funcionais permitiram concluir que a presença dos haplotipos 4 e 7 no promotor de *VCAM1*, associados respetivamente a hemólise grave e AVC pediátrico, levam a um aumento da atividade deste promotor em células endoteliais micro e macrovasculares estimuladas com TNF-α. Verificámos, ainda, *in vitro*, que o principal fármaco usado para o tratamento da doença, a hidroxiureia, diminui a expressão de *VCAM1* induzida por TNF-α.

Globalmente, os nossos resultados reforçam o potencial modulador de *VCAM1* no risco de vasculopatia, cerebral e sistémica, em drepanocitose. A elevada frequência dos haplotipos estudados em crianças de origem subsariana pode afetar a forma como estas reagem às estratégias atuais de prevenção e tratamento do AVC. Os nossos resultados poderão, assim, auxiliar na conceção de planos terapêuticos alternativos para doentes que não respondam ao tratamento com hidroxiureia.

Palavras-chave: drepanocitose, vasculopatia cerebral, moduladores genéticos, AVC, *VCAM1*

ABSTRACT

Sickle cell anemia (SCA) is a genetic disease caused by homozygosity for the HBB:c.20A>T mutation, which results in the production of hemoglobin S (HbS). In hypoxic conditions, HbS polymerizes inside the erythrocytes, altering their morphology into a sickle shape, with increased rigidity and fragility. This triggers complex pathophysiological mechanisms, including inflammation, cell adhesion, oxidative stress, and vaso-occlusion. SCA is phenotypically heterogeneous due to modulation of both environmental and genetic factors. Pediatric cerebral vasculopathy, namely ischemic stroke and silent cerebral infarctions, is one of the most impactful manifestations.

Herein we aimed to identify the modulator effect on the risk and occurrence of pediatric cerebral vasculopathy in SCA, of variants in genes involved, for example, in blood cell-endothelium adhesion (*VCAM1, ITGA4*) or vascular tone (*NOS3*).

We started with candidate gene association studies in a group of 70 SCA children, with previous phenotypical characterization. Subsequently, we analyzed *in silico* the genetic variants with phenotype modulation potential. Finally, we performed functional studies using macrovascular and microvascular endothelial cell models.

We found a positive association between stroke occurrence and the presence of genetic variants in the *VCAM1* promotor (haplotype 7 and rs1409419_T), as well as in *ITGA4* (rs113276800_A and rs3770138_T). *In silico* analyses indicated a potential change in transcription factor binding sites due to the presence of the rs1409419_T variant. On the other hand, functional studies showed that the presence of *VCAM1* promoter haplotypes 4 and 7 – associated with severe hemolysis and pediatric stroke, respectively – increased *VCAM1* promoter activity in TNF-α-stimulated macrovascular and microvascular endothelial cells. Furthermore, we observed that *in vitro* treatment with hydroxyurea – the most frequently used drug for ischemic stroke management and prevention in SCA children – decreased TNF-α-induced *VCAM1* expression.

Overall our results reinforce the *VCAM1* modulation potential on systemic, as well as cerebral, vasculopathy risk in SCA. Being frequent in children of Sub-Saharan ancestry the studied haplotypes may affect how they react to current stroke prevention and treatment strategies. Ultimately, the knowledge we obtained may help devise management strategies for patients non-responsive to hydroxyurea treatment.

Key-words: sickle cell anemia, stroke, genetic modulators, cerebral vasculopathy, *VCAM1*

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ABBREVIATIONS AND ACRONYMS

2,3-DPG – 2,3-diphosphoglycerate

-$\alpha^{3.7kb}$ – 3.7kb deletion in the *HBA2* gene

-$\alpha^{3.7kb}$-thal – α-thalassemia caused by the 3.7kb deletion in the *HBA* gene

α-thal – α-thalassemia

aa – amino acid

AP-2 – activator protein-2

ACS – acute chest syndrome

ATP – adenosine triphosphate

β-thal – beta-thalassemia

BarX2 – homeobox protein BarH-like 2

BMT – bone marrow transplantation

bp – base pair

CAM(s) – cell adhesion molecule(s)

CBF – cerebral blood flow

CBT – chronic blood transfusion (therapy)

cGMP – cyclic guanosine monophosphate

$CMRO_2$ – cerebral metabolic rate of oxygen utilization

CO – carbon monoxide

CO_2 – carbon dioxide

CVD – cardiovascular disease

CSSD (trial) – *Cooperative Study of Sickle Cell Disease*

DAMP – damage-associated molecular pattern molecule

EC(s) – endothelial cell(s)

ECM – extracellular matrix

EMA – European Medicines Agency

eNOS – endothelial nitric oxide synthase (=NOS3)

ENPP1 - ectonucleotide pyrophosphatase/phosphodiesterase family member-1 gene

EVI-1 – ecotropic virus integration site-1

Fe^{2+} – ferrous ion

Fe^{3+} – ferric ion

FBS – fetal bovine serum

FDA – Food and Drug Administration

GATA-1 – GATA binding protein-1

GOLGB1 – Golgin subfamily B member 1 gene

GWAS – genome-wide association studies

Hap – haplotype

Hb – hemoglobin

HbA – adult hemoglobin (normal)

HBA(1 or ***2)*** – α-globin gene (1 or 2)

HBB – β-globin gene

HbC – hemoglobin (variant) C

HbF – fetal hemoglobin

HBG – gamma hemoglobin gene

HbS – hemoglobin S (sickle)

HBEC(s) – human brain endothelial cell(s)

***HMOX1* –** heme-oxygenase 1 gene

HO-1 – heme-oxygenase 1

HPFH – hereditary persistence of fetal hemoglobin

HS – hypersensitive site

HSCT – hematopoietic stem cell transplant

HU – hydroxyurea

I/R – ischemia/reperfusion

ICA – internal carotid artery

ICAM-1 – intercellular adhesion molecule 1

IL-1β – interleukin 1β

ITGA4 – integrin α4 gene

LCR – locus control region

LDH – lactate dehydrogenase

LR-PCR – long-range PCR

LPS – lipopolysaccharide

metHb – methemoglobin

mRNA – messenger RNA

MCA – middle cerebral artery

MCS – multispecies conserved sequence

MCV – mean corpuscular volume

MCH – mean corpuscular hemoglobin

MRA – magnetic resonance angiography

MRI – magnetic resonance imaging

NADH – nicotinamide dehydrogenase

NADPH – nicotinamide adenine dinucleotide phosphate dehydrogenase

NF-κB – nuclear factor kappa B

NGS – next-generation sequencing

NO – nitric oxide

NOS – nitric oxide synthase

***NOS3* –** endothelial nitric oxide synthase gene (=eNOS)

O_2 – oxygen

Oct-1 – octamer-binding transcription factor-1

OEF – oxygen extraction fraction

PCR – polymerase chain reaction

PON1 – paraoxonase-1 gene

PS - phosphatidylserine

REACH (trial) – *Realizing Effectiveness Across Continents with Hydroxyurea*

RT-qPCR – real-time quantitative PCR

RBC(s) – red blood cell(s)

ROS – reactive oxygen species

SCA – sickle cell anemia

SCD – sickle cell disease

SCI – silent cerebral infarct

SIT (trial) – *Silent Cerebral Infarct Transfusion*

SNP(s)– single nucleotide polymorphism(s)

SSRBC(s) – irreversibly sickled red blood cell(s)

STOP (trial) - *Stroke Prevention Trial in Sickle Cell Anemia*

SWAY – *Sickle Cell World Assessment Survey*

SWiTCH (trial) – *Stroke with Transfusions Changing to Hydroxyurea*

TAMMV – time-averaged mean of maximum velocity

TCD – transcranial Doppler (ultrasonography)

Thal - thalassemia

TIA – transient ischemic attack

TLR4 – Toll-like receptor 4

TNF-α - tumor necrosis factor-α

TSP – thrombospondin

TWiTCH (trial) – *TCD With Transfusions Changing to Hydroxyurea*

***VCAM1* –** vascular cell adhesion molecule-1 gene

VCAM-1 – vascular cell adhesion molecule-1

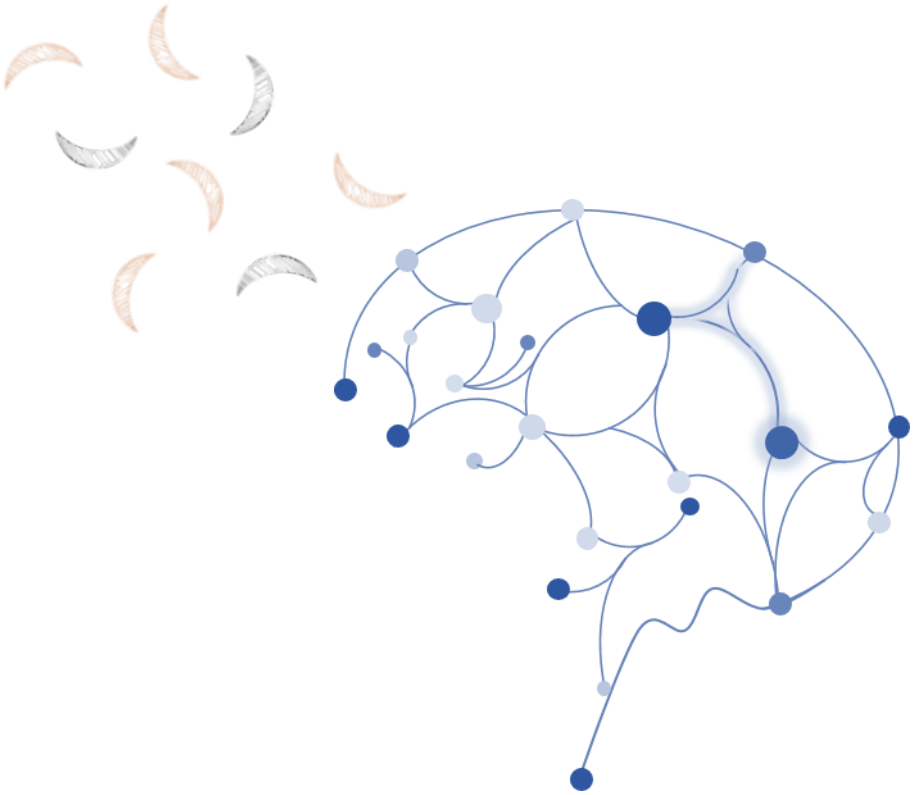
VLA-4 – very late antigen 4

VNTR – variable number of tandem repeats

VOC – vaso-occlusion (vaso-occlusive)

vWF – von Willebrand factor

WBC(s) – white blood cell(s) (=leukocytes)



General Introduction and Thesis Objectives

Part I

Chapter 1

INTRODUCTION AND OBJECTIVES

1. INTRODUCTION

Sickle cell disease (SCD) is one of the most common hemoglobin disorders worldwide. It has been extensively studied in terms of molecular mechanisms as well as clinical manifestations. Despite being a result of a point mutation that affects a single gene, SCD behaves as a highly complex and multifactorial disease. The abnormal hemoglobin that results from the causal mutation leads to severe changes in red blood cell function and properties, which in turn affect blood circulation and interactions with the vascular wall. To study and understand SCD pathophysiology it is thus important to first consider the normal context of all these elements. Hence, an overview of the fundamental elements of normal human hemoglobin, red blood cell, and vascular properties functions is provided in sections 1.1 to 1.3. SCD and especially its more severe form, sickle cell anemia (SCA), is then addressed in section 1.4. Given its impact on patients with SCA, an emphasis is given to the pediatric cerebrovascular disease. Their pathophysiology and potential severity modulation by genetic modifiers are specifically discussed in sections 1.4.4 and 1.4.5.

1.1. HUMAN HEMOGLOBIN

1.1.1. Hemoglobin Structure and Function

The first description of the crystallographic structure of human hemoglobin (Hb) was provided by Perutz in 1960 [1], for which he was awarded the Nobel Prize in Chemistry in 1962. Since then more than 1800 Hb variants have been identified, most of them innocuous, while others have been associated with disease.

Human hemoglobin (Hb) molecules constitute a group of closely related molecules, where symmetrically paired dimers of α-globin-like and β-globin-like polypeptide chains are arranged in a tetrameric structure (Fig. 1.1). The Hb heterotetramer, located inside RBCs, is responsible for O_2 delivery to the tissues and return transport of CO_2 from there to the lungs. Each of the two α-globin polypeptide chains (141 amino acids per chain) and each of the two β-globin polypeptide chains (146 amino acids per chain) have a large central space into which a prosthetic heme group, an iron protoporphyrin IX molecule, is bound by noncovalent forces [2,3]. The iron atoms are mainly in the ferrous state (Fe^{2+}). They are coordinated, in one plane, to four pyrrole nitrogen (N) atoms and an imidazole N atom of the invariant histidine amino acid at position 8 of the "F"-helix, while in the opposite plane (regarding the porphyrin plane) to the histidine residue [3].

The Fe^{2+} form is fundamental for O_2 transport but also shows a high affinity to carbon monoxide (CO) and nitric oxide (NO), namely through the reversible binding of gases to the four ferrous iron atoms in globin tetramers [2,3]. O_2 binds reversibly to those Fe^{2+} ions with a cooperative interaction between binding sites that further facilitates combination with other O_2 molecules [2]. For example, at a partial pressure of O_2 of 100 mm Hg the Hb inside RBCs becomes fully saturated with oxygen. CO binds to the ferrous ion even more firmly which is why coal gas poisoning is so severe – once carboxyhemoglobin forms O_2 cannot displace CO. The main factors that affect the effectiveness of oxygen transport system in the body are: (i) the adequate blood oxygenation in the lungs, (ii) the rate and distribution of blood flow, (iii) the oxygen-carrying capacity of the blood (Hb concentration), and (iv) the Hb-O_2 affinity to allow unloading O_2 in the peripheral capillaries [2].

In addition to the O_2-transport-related functions, NO/Hb interactions are also essential, given the physiological importance of NO, namely in vascular tone regulation, cell-cell interactions, and neural functions [4]. NO is a cell-signaling molecule that acts via soluble guanylyl cyclase production of cyclic GMP and through other mechanisms. It can react with oxyhemoglobin to produce methemoglobin, with ferric iron and nitrite ions, but also with deoxyhemoglobin to form nitrosyl(heme)hemoglobin (NO-hemoglobin), with the NO ligated to the ferrous ion atom (see section 1.1.3.1). O_2-related and non-related functional properties of Hb are clinically important (e.g., due to hypoxia as a consequence of abnormalities in O_2 transport; or due to NO depletion resulting from high Hb levels, localization, or even of cell-free Hb.

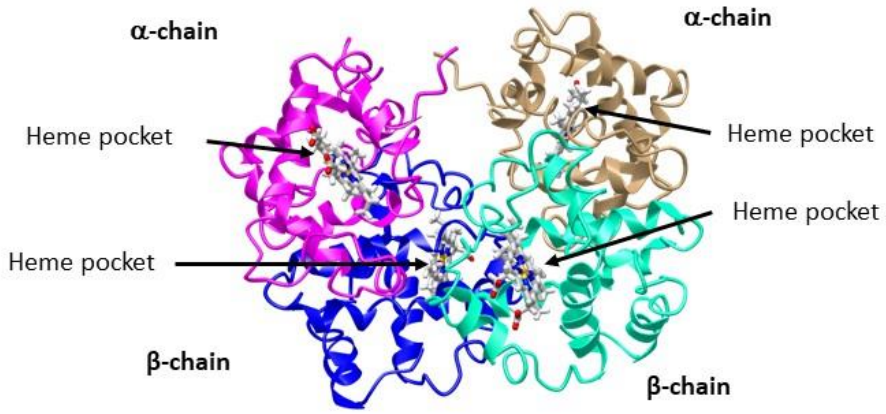


Figure 1.1. ***Crystalographic structure of human hemoglobin A.*** The tetramer is constituted by two α-chains (in pink and brown), two β-chains (in blue and green), and four heme groups (arrows). Obtained from the Molecular Modelling Database, 2H35 coordinates [5,6], using the iCn3D software [7].

1.1.2. Transcriptional Regulation of Hemoglobin Switching

In normal adults, hemoglobin A ($\alpha_2\beta_2$) is the predominant form inside RBCs, accounting for approximately 97% of Hb molecules [3]. The smallest fractions correspond to HbA_2 ($\alpha_2\delta_2$) accounting for ~2%, and fetal hemoglobin (HbF, $\alpha_2\gamma_2$), accounting for ~1% [2,3,8,9]. These different Hb tetramers and their heterogeneous distribution results from differential expression of globin genes throughout development, from the embryo stage through adulthood (Fig. 1.2). Hb synthesis is controlled by two multigene clusters on chromosome 16, region p13.3 (α-like genes), and on chromosome 11, region p15.4 (β-like genes) (Fig. 1.2), where the genes are organized in the same transcriptional orientation and according to the order of expression during development [8–10]. The embryonic ε-gene (β-like) is expressed in the first eight weeks of gestation by erythroblasts existing in the yolk sac, together with the zeta-gene (α-like) to form embryonic hemoglobin – Hb Gower I ($\xi_2\varepsilon_2$), Hb Gower II ($\alpha_2\varepsilon_2$) and Hb Portland ($\xi_2\gamma_2$)[2,8,10]. The first major Hb switching event occurs during fetal erythropoiesis, in the liver. Fetal hemoglobin (HbF, $\alpha_2\gamma_2$) starts being produced, as a result of the paired expression of γ-globin and α-globin genes.

There are two human γ-globin genes, $^{A}\gamma$ and $^{G}\gamma$, which differ from each other in one amino acid at codon 136. A second Hb switching event occurs after the 2nd trimester, as a result of a slow and continuous increase in β-globin gene expression. This increase continues until the perinatal period, where it becomes predominant and bone marrow hematopoiesis is established [2,8]. From the age of 3 years onwards hemoglobin A (HbA, $\alpha_2\beta_2$), the tetramer resulting from α- and β-globin genes' expression, is the major (>95%) component of total Hb in normal individuals, while HbF plunges to 1-2%.

The differential expression of globin genes during ontogenesis is controlled by major regulatory elements – the locus control regions, LCR – that act on the transcriptional status of that particular genomic/chromatin domain. Enhancers of the β-globin genes are also included in the β-globin gene cluster, specifically in its 3'-terminal flanking region [9].

The LCR of the β-globin gene domains is composed of several modules co-localized with individual hypersensitivity sites of DNase I (DHS2 to DHS5) [11]. DHS2 to DHS4 constitute erythroid-specific enhancers, with the most powerful enhancer being located at DHS2 [11–13]. GATA1, NF-E2, AP1, and CP2 are different transcription factors for which binding sites mapped in DHS2-4 were identified [12,14]. The LCR is mainly a block of tissue-specific enhancers that act, either through direct enhancer-promoter interaction by looping the chromatin fragment between them (as in the proximity to the embryonic ε-gene), or through the formation of short-lived alternative complexes between the LCR and the gene promoters (as in the case of the genes δ and β) [8]). The knowledge of α- and β-globin genes, respective domains, and particularly of the LCR of the β-globin gene domain has played an extremely important role in the design of the currently accepted model of domain organization of the genome. However, the exact mechanism of Hb switching/differential gene expression is still unknown.

The Hb switching events, where differential β-like globin gene expression occurs at different stages of ontogenesis, namely HbF to HbA, are of the utmost importance for understanding the pathophysiology of hemoglobin disorders (hemoglobinopathies). The abnormal phenotypes are only apparent after β-globin prevails over γ-globin gene expression, hence only in early infancy and thereafter [2,8]. HbF level (or its related phenotype F-cell number) is the major modifier of clinical severity in the Hb disorders

(hemoglobinopathies) that affect the β-globin chain, namely SCA. HbF level is considered a quantitative trait and has been shown to be regulated by loci unlinked to the β-globin cluster. Those loci, designated quantitative trait loci (QTL) include the *Xmn I* site upstream of the *HBG2* gene (rs7482144, on chromosome 11p15.4), *KLF1* (on chromosome 19p13.13), *BCL11A* (on chromosome 2p16.1), and intergenic polymorphisms *HBS1L-MYB* (on chromosome 6q23.3) [15–17]. Regardless of this evidence of genetic control of HbF expression/silencing, the knowledge of critical events regulating globin synthesis and switching that occur early in erythroid progenitor cells following a developmental clock remains incomplete.

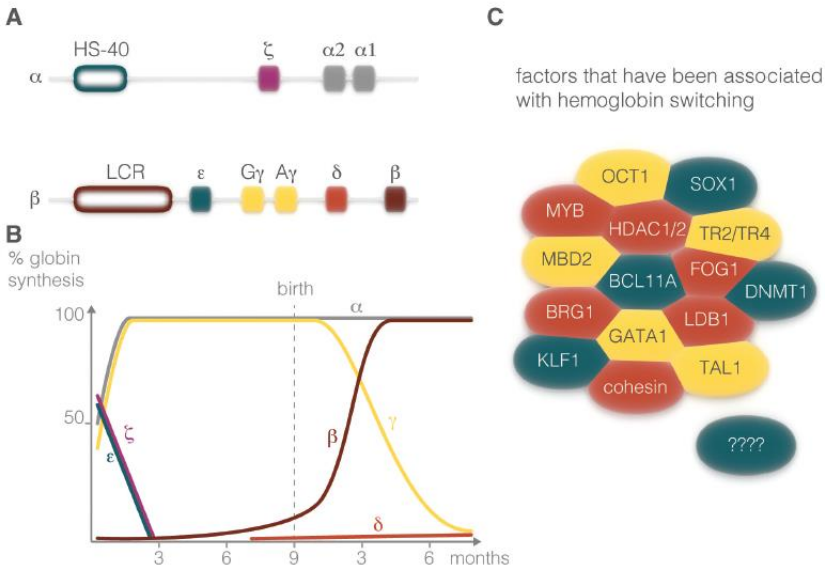


Figure 1.2. ***Diagram of the genomic structural organization of the human globin genes, their temporal expression, and factors that affect Hb switching.*** *(A)* The gene order of the α- and β-globin genes, on chromosomes 16 and 11, respectively, is represented; LCR: locus control region (not to scale). (B) The different types of globin chains produced from each site are shown at each temporal stage as indicated on the timeline at the bottom. (C) Representation of candidate proteins involved I the regulation of fetal Hb to adult Hb switching. Reproduced from Rivella, 2015 [18], with permission of *Haematologica* journal, under a CC BY-NC-ND license.

1.1.3. Hemoglobinopathies

The Hb disorders, or hemoglobinopathies, are the most common genetic diseases, with more than 300,000 severely affected individuals born yearly worldwide [19]. The underlying genetic defects affect Hb production through altered Hb function, stability, or Hb levels. Frequent in tropical and subtropical areas, due to a close association between carrier status and protection against malaria [20], Hb disorders exhibit phenotypic heterogeneity, despite the monogenic mode of inheritance. Sickle cell anemia was the first to be described as a molecular disease by Linus Pauling in 1949 [21]. Subsequently, Ingram identified a single amino acid substitution in the β-globin chain as the etiologic molecular mechanism [22]. Henceforth, the studies of the etiologic molecular defects of thalassemias further strengthened the understanding of mechanisms underlying disease [23].

Hemoglobinopathies can be divided into three classes according to the effect of the underlying mutation: (i) structurally abnormal Hb variants (qualitative defects), that arise from an altered polypeptide chain, with an unaffected rate of synthesis; (ii) thalassemias (quantitative defects) where the mutation leads to an impairment in the synthesis and/or stability that results in an imbalance of the globin chains available; and (iii) hereditary persistence of fetal hemoglobin (HPFH), due to absence of γ to β globin switch that leads to high levels of HbF ($\alpha_2\gamma_2$) in adulthood, and milder phenotypes of β-hemoglobinopathies [8,24].

Hb abnormalities also lead to changes in the biomechanical properties of RBCs' (see section 1.2). The pathophysiology of these diseases is therefore complex and affected not only by coding sequences but also by splice sites, 3' and 5' untranslated regions, proximal gene promoters, or even by distal regulatory regions, like enhancers or suppressors [25].

The HbF level (or the related phenotype F-cell number) is a major modifier of clinical severity in hemoglobinopathies resulting from an abnormal β–globin chain [25,26].

1.1.3.1. Abnormal Hemoglobins

Amino acid substitutions in the α- or β-globin chain result in Hb variants. These may be detected after a physical examination and routine laboratory testing, which would explain the high number of Hb variants described to date (see HbVar, http://globin.cse.psu.edu/hbvar/menu.html). Some variants have altered surface charge that allows them to be detected through the electrophoresis or chromatography techniques routinely used throughout Europe and North America [27]. Variants with impaired Hb-O2 affinity that lead to erythrocytosis, cyanosis, or even benign skin tone changes, depending on the degree of affinity impairment, have also been described [27]. The most clinically relevant abnormal Hb is hemoglobin S (HbS), due to its role in sickle cell disease (SCD) (see section 1.4). Hemoglobin C (HbC) and E (HbE) are also common and, like HbS, are the result of amino acid substitutions in the β-globin polypeptide chain. All of these are associated with intravascular hemolysis and cause chronic hemolytic anemia, with varying degrees of severity [27].

1.1.3.2. Thalassemias

A diminished rate in α- or β-globin chain synthesis leads to α-thalassemia (α-thal) or β-thalassemia (β-thal), respectively. The main syndromes are thalassemia major (transfusion-dependent), thalassemia intermedia (non-transfusion dependent thalassemia), and thalassemia minor (carrier state of α- or β-thal).

α-thal occurs whether as a result of deletions or mutations in the α-globin genes, although the deletional mechanism is the most frequent (Fig. 1.3). Besides the *HBA2*

and *HBA1* genes (on each chromosome), the normal cluster, commonly designated αα, also includes four non-coding highly conserved sequences – the multispecies conserved sequences (MCS), MCS-R1 to MCS-R4 [19]. The number of α-globin genes missing or inactive is determining for disease severity. Loss of all four copies leads to the production of Hb Bart, due to a total absence of α-chain synthesis, a condition incompatible with life that results in intrauterine death due to hydrops fetalis. Deletion of three copies of the gene causes HbH disease, a moderately severe microcytic hypochromic anemia (Hb concentration = 7.0-11.0 g/dL) with splenomegaly. HbH is composed of four β-chains and this tetramer can be detected inside the patients' RBCs by electrophoresis or in reticulocyte preparations. In fetal life, γ- instead of β-chains form the tetramers.

When only one or two copies of the α-globin genes are lost, α-thal traits occur. These are usually not associated with anemia, but cause low mean corpuscular volume (MCV) and/or mean corpuscular hemoglobin (MCH). Definitive diagnosis may only be achieved through molecular genetic testing.

The most common molecular mechanism causing α-thal is a 3.7 kb deletion that leads to the formation of only one hybrid α gene (the $-\alpha^{3.7kb}$ gene) [10,19]. Homozygosity or heterozygosity may occur, as well as coinheritance with a β-globin gene mutation, in which case, the $-\alpha^{3.7kb}$ gene has been shown to act as a disease modifier, with ameliorating effects (see section 1.4.5).

Other less frequent forms of α-thal arise from point mutations that lead to altered gene function or unstable chain (e.g., Hb Constant Spring). α-thal associated with mental retardation results from large deletions of the terminal region of the short arm of

chromosome 16, mutations in the ATR-16 gene (or ATR-X gene, on chromosome X), that controls α-globin gene transcription [28].

When no β-chain (β^0) or only small amounts of it (β^+) are synthesized, this may lead to β-thal major. The α-chains produced in excess precipitate inside erythroblasts and immature RBCs causing ineffective erythropoiesis. The higher the excess, the more severe the anemia. This effect may be ameliorated by an increase in the production of γ-chains.

Unlike α-thal, point mutations are the main causal mechanisms in β-thal, whether within the gene cluster, the promoter, or enhancer regions [10,29]. Thalassemia major and thalassemia intermedia usually result from compound heterozygosity of two different parental mutations, each affecting β-chain production. Deletional events within the β gene, δ and β genes, or even of δ, β and γ genes may occur. δβ-thal involves the absence of δ and β chain synthesis. The heterozygous state is associated with an increase in HbF production (5-20%), resembling thalassemia minor, while in the homozygotes only HbF is present, and the hematological phenotype is similar to thalassemia intermedia.

Unequal cross-over events result in the production δβ-fusion genes and consequently a δβ-fusion chain (e.g., Hb Lepore). This is synthesized inefficiently and eliminates the production of normal δ and β-chains. Homozygosity for this defect causes thalassemia intermedia and heterozygosity leads to thalassemia trait.

Hereditary persistence of HbF is a group of genetic conditions caused by (i) deletions or unequal cross-overs that affect β and γ-chain production, or (ii) point mutations upstream from the γ-globin genes or in their regulatory genes [16,30].

β-thal minor, or β-thal trait, is a common, but usually asymptomatic abnormality, that may manifest with a microcytic hypochromic mild anemia (low MCV and MCH, Hb 10.0-12.0 g/dL) and a high RBC count (>5.5 x 10^{12}/L). The clinical severity is usually higher than the one of α-thal trait. The diagnosis can be confirmed by the presence of an increased HbA_2 (>3.5%). Definitive diagnosis is critical for disease prevention since a couple where both are carriers of the β-thal trait have a 25% risk of affected offspring.

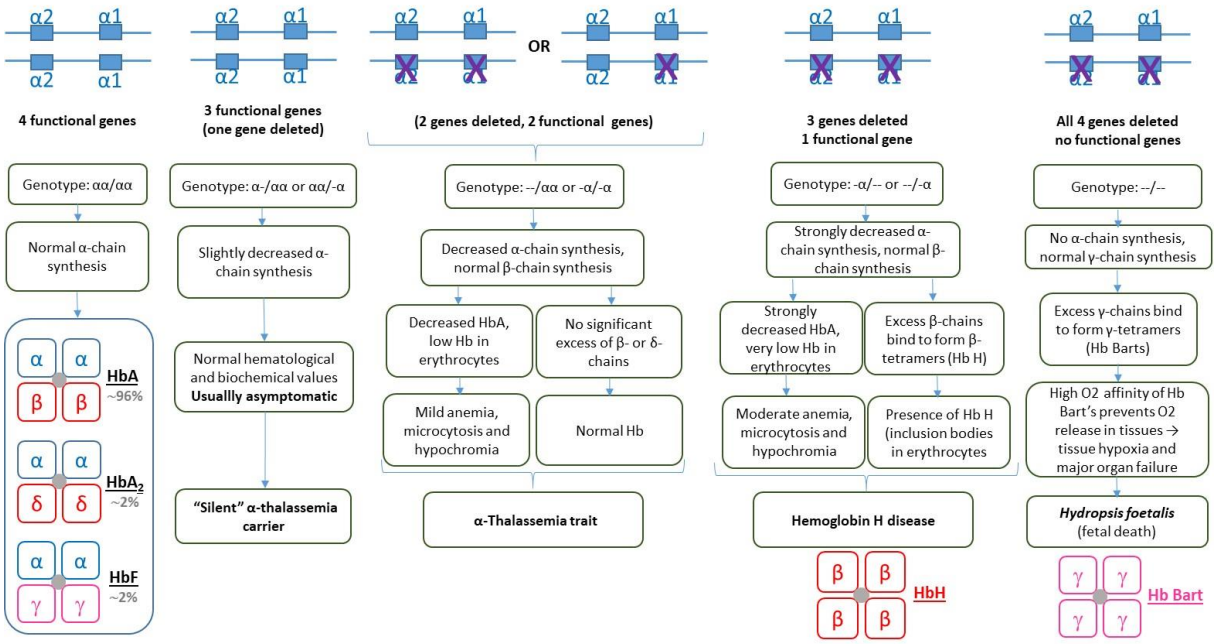


Figure 1.3. ***Pathophysiology of deletional α-thalassemia.*** The possible mechanisms of α-globin gene(s) deletion are depicted. The biochemical, hematological, and clinical consequences are also indicated. α: alpha-globin chain; β: beta-globin chain; δ: delta-globin chain; γ: gamma-globin chain; α2: *HBA2* gene; α1: *HBA1* gene; HbA: hemoglobin A; HbA_2: hemoglobin A2; HbH: hemoglobin H; Hb Bart: hemoglobin Bart. *(© Marisa Silva, 2020*).

The association between β-thal trait with Hb defects has varying consequences. A combination with the HbE trait, for example, usually causes a transfusion-dependent

thalassemia major syndrome or, in some instances a thalassemia intermedia. β-thal trait associated with HbS trait leads to a sickle cell anemia phenotype (*see section 1.4*) instead of a thalassemia clinical picture. A combination with a HbD trait results in microcytic hypochromic anemia with variable degrees of severity.

1.2. RED BLOOD CELLS

Hemoglobin can only perform its role successfully through efficient transport by RBCs, the main cells in the blood circulation. RBCs have very particular characteristics in terms of cell content, as well as membrane structure and composition, which confer them specific properties and functions. Devoid of all organelles and most intracellular machinery during maturation, they evolved to maximize their Hb content and O_2 delivery to all periphery tissues. RBCs also interact with other blood cells and with cells of the vascular walls, thus affecting blood flow and tissue perfusion.

As with all other blood cells, RBCs are produced during hematopoiesis, a highly regulated and complex process that occurs in the bone marrow. Approximately 10^{12} new RBCs are produced daily in the human body, through erythropoiesis (Fig. 1.4), beginning in the bone marrow's erythroid niche, progressing from a pronormoblast to a late normoblast which, still in the bone marrow, suffers nuclear extrusion resulting in a reticulocyte. This cell, which still contains ribosomal RNA and synthesizes Hb, can be considered an immature RBC but with a slightly larger size and still containing some RNA. After 1-2 days in the peripheral blood circulation, it matures and finally loses its RNA content. The result is an enucleated biconcave discoid cell, approximately 8 μm in diameter, with specialized functions and particular properties. These cells are required to pass repeatedly through the intricate circulatory network, especially in

capillaries with a minimum diameter of 3.5 μm, to maintain Hb in a ferrous state and to maintain osmotic equilibrium, regardless of its Hb concentration. Hence, normal RBCs are highly flexible. They are also able to generate energy in the form of adenosine triphosphate (ATP) – through the anaerobic glycolytic Embder-Meyerhof pathway – which provides energy to maintain RBC volume, shape, and flexibility [31]. The same pathway allows for the production of NADH that is necessary for methemoglobin reductase to reduce functionally dead methemoglobin (containing ferric ions) to functionally active reduced Hb (containing ferrous ions). The sidearm of the Embder-Meyerhof pathway (the Luebering-Rapoport shunt) generates 2,3-diphosphoglycerate (2,3-DPG), which is important in Hb-O_2 affinity regulation [31,32].

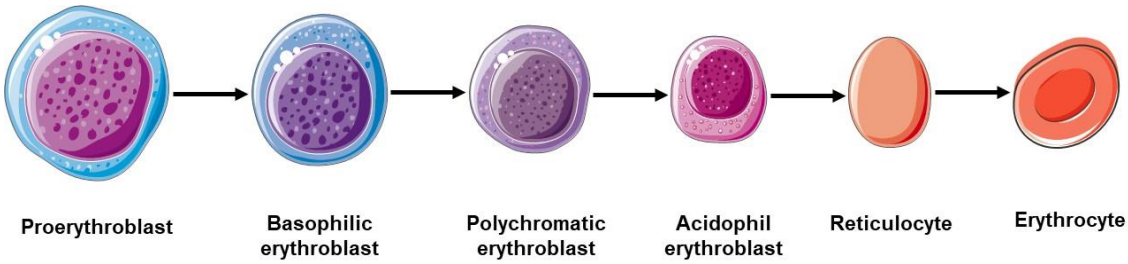


Figure 1.4. ***Schematics of erythropoiesis*** *(simplified).* Proerythroblasts differentiate from the burst forming unit erythroid (BFU-E) and colony-forming erythroid (CFU-E) progenitor cells (not shown) that can only be identified by colony assays. Progressive maturation then occurs from basophilic erythroblasts, polychromatic erythroblasts, and acidophil erythroblasts. The latter undergo enucleation giving rise to reticulocytes, which are released into the blood circulation and maturing further into erythrocytes (or RBC). Created using ServierMedArt, under a CC-BY-3.0 license.

The predominance in the blood flow and specific flow-affecting properties confer RBC a central role in hemorheology and hemodynamic behavior. Hemorheology addresses

the flow and deformation of blood and its components, which play an important role in the microcirculation and ultimately in tissue perfusion [33,34].

The RBC properties of deformability, self-aggregability, and potential adherence to vascular endothelium (henceforth designated as RBC adherence) are especially important for O_2 transport, through large- and small vessels, as well as delivery to tissues [33,35,36]. Interactions between the membrane skeleton, membrane proteins, and lipid bilayer are determinant for those properties, namely for allowing the RBC to deform, under shear stress from the blood flow, and later regain its typical biconcave discoid shape. Biochemical components of RBCs' membrane, like sialic acid, phospholipids, band-3 clusters, and adhesion molecules, as well as RBC cellular content, govern those flow-affecting properties [35].

Aggregability relates to RBCs' ability to form multicellular aggregates, shaped as stacks of coins (*rouleaux*), in the presence of plasma proteins or other macromolecules. It results from the balance between aggregating and disaggregating forces: repulsion between negatively-charged cells; cell-cell adhesion induced by the presence of plasma proteins; and disaggregating fluid shear forces [34,35]. RBC aggregation may contribute to vascular resistance to flow, however, it may also be accompanied by RBC migration to the center of the blood vessel, especially in the venous circulation. This will induce the formation of an RBC-free layer near the vessel walls. The result of those opposing effects on vascular resistance – increased viscosity in the center and reduced viscosity near the walls of the blood vessel – may vary according to the type and size of the blood vessel. Dispersion of the RBC aggregates, before entering the capillaries, usually occurs simply by the action of normal flow and is critical for effective tissue perfusion. Conversely, low-flow conditions do not allow for easy RBC disaggregation and favor the formation of larger, more resistant aggregates,

further enhancing blood viscosity, subsequent resistance to flow, and reduced flow rate. Increased aggregability may potentially impede or block blood flow in small blood vessels which, in turn, may induce a tissue perfusion reduction, ischemia, and ultimately infarct [37]. The positioning of RBC aggregates near the vessel walls may also lead to decreased O_2 diffusion through the cell-free layer to the vessel wall [38]. Non-hemodynamic effects of RBC aggregability include (i) activation of endothelial cells (EC) due to increased shear stress at the vessel wall; (ii) promotion of platelet migration to the vessel wall and their interaction with ECs; and (iii) facilitation of WBC margination to the vessel walls and their adhesion to the endothelium [39].

The RBCs' ability to adapt their shape to flow conditions thus minimizing their resistance to blood flow, enables these cells (with an average diameter of 8 μm), to cross capillaries with diameters lower than 3-5 μm. This essential deformability property allows for maintaining a constant O_2 supply to the tissues. When diminished, it may lead to impaired O_2 perfusion and delivery to the peripheral tissues. In large vessels, RBCs' deformability is also important as it enables them to change to an ellipsoid shape and to reduce the bulk viscosity in the macrovasculature while maintaining blood fluidity. RBCs' survival may also be strongly determined by their deformability, especially in the spleen. The very small endothelial slits (0.5-1.0 μm) that RBCs traverse in this organ act as a filter. Aging RBCs with reduced deformability are therefore retained inside the organ, ultimately leading to splenic sequestration [40]. Additionally, structural changes in RBCs, namely in cytoskeleton or intracellular viscosity, impact deformability, and may subsequently alter blood flow, particularly in low-flow states [35].

In normal conditions, RBC-EC adherence is negligible, whereas in pathological states changes in RBC membrane properties make them adherent to ECs [37,41]. This abnormal

adherence or adhesion may, in turn, result in blocked capillaries and is a determinant mechanism in vascular occlusion (or vaso-occlusion), especially in the microvasculature. This has been considered an underlying pathophysiological mechanism in diseases where RBC abnormalities are present and seems to correlate with the occurrence and severity of vaso-occlusion as a whole [41–43]. In large vessels, the sequestration of RBC, due to increased adhesive properties, may result in impairment of local flow patterns and shear stress and, subsequently to endothelial activation [44]. Furthermore, oxidized RBCs, like the ones found in hemoglobinopathies or diabetes, may apply oxidative stress on ECs and also contribute to endothelial activation [40,45]. Overall the RBC flow-affecting properties – aggregability, deformability, and adherence – have been identified (individually or in conjunction) as underlying pathophysiological mechanisms in several diseases, namely cardiovascular diseases, diabetes, and hemoglobinopathies (SCD and thalassemia).

As noted earlier, the biochemical membrane components and cellular content of RBCs impact their flow properties. Any changes in these factors will potentially induce variations in hemodynamic behavior, whether acting on each property independently or causing multiple effects on all properties simultaneously. Biochemical changes may also have a vascular effect regardless of their influence on RBC rheology [35]. The RBC membrane is a lipid bilayer linked to an underlying membrane skeleton, where transmembrane proteins interact both with the lipid core as well as with skeletal proteins (Steinberg, 2016). Glycophorins (GP, A to D), the Rh proteins, transport proteins, (e.g., band 3, PIEZO1, the Gardos channel), Ca2+ ATPase, and Mg2+ ATPase are among the transmembrane proteins that can be found in RBCs. Together with phosphatidylserine (PS), spectrin (in the cytoskeleton), and adhesion molecules (e.g., ICAM-4, Lu/BCAM), they strongly affect RBC properties. On the other hand,

the Hb concentration (mean corpuscular hemoglobin concentration, MCHC) is the major intracellular intervening element on RBC hemorheological properties. Sialic acid is the main contributor to the surface charge of the RBC membrane, hence its surface level is the major factor in aggregation, and conversely, RBC aggregation is highly sensitive to changes in the surface sialic acid level [46]. It highly affects glycophorins, namely GPA, which in turn impact RBC membrane surface charge and the membrane glycocalyx composition [47]. Phospholipid composition is also essential for RBC aggregation, namely through interaction with the cytoskeleton [48]. Phosphatidylcholine and sphingomyelin compose the outer leaflet, whereas the inner leaflet contains phosphatidylethanolamine, phosphatidylserine (PS), and phosphoinositide [49,50]. PS translocation from the inner membrane leaflet to the outer leaflet (PS exposure or externalization) allows for RBC surface binding to different proteins and subsequent RBC-EC adhesion. During aging and under certain pathological conditions, PS exposure increases concentration on the external surface. The process is mediated by Ca^{2+}-dependent scramblase by lysophosphatidic acid (LPA), and phorbol-12 myristate-13-acetate (PMA) in a Ca^{2+}-dependent and a Ca^{2+} independent manner [51]. In SCD, PS exposure upon deoxygenation is linked to Ca^{2+} influx that activates the Gardos channel activation and signals eryptosis (RBC cell death) [51,52]. Additionally, PS exposure increases adhesion (activating ECs and contributing to inflammation) and promotes coagulation (through direct platelet activation or, by hemodynamic change at the vessel wall indirectly leading to EC/platelet interaction) [53,54]. Expression of exposed PS ligands, like PS-receptor on ECs, is (directly or indirectly) upregulated by lipopolysaccharide, cytokines, hypoxia, and heme [55].

Anion channels and exchanger proteins, like PIEZO1, the Gardos channel, and band-3 are essential for maintaining RBC volume homeostasis, as well as flexibility, stability,

and deformability of RBCs [47,56]. For instance, abnormal K^+ loss due to Gardos channel activation causes RBC dehydration, thereby enhancing mechanisms like RBC sickling in SCD [57] (*see section 1.4.2*), while the band 3 protein plays a role in RBC-EC adhesion, namely in oxidative stress states where its clustering induces RBC adhesion to ECs[45]. Although RBC membranes contain several adhesion molecules, in normal conditions RBC-EC adhesion is minimal, and their role in these conditions may be related to hematopoiesis and clearance of RBC [41]. However, in abnormal RBCs, like sickle cells and stress reticulocytes, adhesion molecules like VLA-4, and CD44 may be found. In a damaged endothelium, CD44 can mediate RBC attachment to the vascular extracellular matrix, through fibronectin binding, and promote thrombus formation [54,55]. Besides membrane components, cytoskeleton elements and cellular content also affect RBC properties. Therefore, spectrin, a cytoskeleton component, particularly the spectrin state, is a major determinant of RBC deformability. In oxidative states, spectrin cross-linking and/or subsequent membrane rigidity are likely to occur, as in hemoglobinopathies and other inflammatory conditions [49].

Finally, Hb determines RBC intracellular viscosity, subsequently affecting cell deformability. The O_2 transport role of RBCs is intimately dependent on this molecule and therefore is largely affected by Hb content and structure. High Hb concentrations and/or significant polymerization, due to abnormal Hb structure (see *section 1.1*), result in increased intracellular viscosity and, consequently, in decreased deformability [46].

In summary, RBC properties may significantly affect hemodynamics but may also have non-hemodynamic effects, and are thus critical in controlling circulatory processes. Those properties may exert their effects simultaneously, synergistically or independently, or even remain unaltered in an independent manner. Additionally, biochemical factors may affect each property individually, several properties

simultaneously, and further affect other cells in the vascular system, without acting on RBC rheology. In pathological conditions, abnormal RBCs may modulate shear stress, lower flow rate, promote endothelial adhesion of RBC (and of leukocytes) to the vessel wall, induce vaso-occlusion and facilitate platelet activation and hypercoagulability.

1.3. VASCULAR ENDOTHELIUM

Endothelial cells (ECs) provide the coating (endothelium) of the luminal surface of all the vessels that constitute the intricate circulatory network of the body. More than being a simple passive barrier, this interface between the blood and underlying tissues plays an essential role in a multitude of physiological functions in the cardiovascular system, including regulation of vascular tone and growth, fluid and solute exchange, inflammatory response, hemostatic balance, platelet-leukocyte interaction, cell proliferation, and angiogenesis [58–61]. With the ability to act in sensory and effector capacities, the endothelium responds to humoral, neural, and mechanical stimuli through the synthesis and release of vasoactive substances [60–62]. Vascular dilation occurs as a response to endothelium-derived relaxing factors (EDRF), namely NO, as well as the endothelial-derived hyperpolarizing factor (EDHF), [63–66] while vascular contraction may be induced by the release of endothelium-derived contracting factors (EDCF), such as endothelin, thromboxane A2, angiotensin II, and O_2^- [67–70]. ECs may also produce growth inhibitors or promoters, such as heparin and heparin sulfates, platelet-derived growth factors, and thrombospondin. NO, endothelin, and angiotensin may also affect vascular growth regulation [71,72].

Albeit constituting an extensive part of the vasculature, the endothelium is not a homogeneous tissue of all-identical cells. From a phenotypical point of view, ECs are

highly heterogeneous and vary in structure and function, space and time, and health and disease [58]. Those differences are in line with the multitude of physiological functions of the endothelium, namely the demands of the underlying tissues, and also the need for adaptation and survival in different environments across the human body[59]. The basic functions of ECs are fundamentally the same, regardless of vessel size: to provide a non-thrombogenic surface to which blood cells fail to adhere; to mediate the passage of nutrients and other solutes from the blood to the tissues; to produce vasoactive agents that maintain vessel patency and prevent platelet aggregation; to maintain the vessel lumen open by growing as a monolayer firmly adherent to the basal membrane of the vessel wall [73,74]. However, ECs differ in terms of intercellular tight junctions, with the endothelium being present without fenestrations or perforations (as seen in muscle, lung, central nervous system, for example), with intracellular fenestrations or perforations (like in kidney, intestinal villi, and some endocrine glands) or with intercellular fenestrations or gaps (as described in liver and spleen) [75,76]. ECs may also diverge in terms of morphology, mediator release, antigen presentation, or stress response, and individual cells can also differ from the immediately adjacent endothelium [58]. ECs of large and small vessels differ in their morphology, with large arteries and veins showing a tightly packed layer of polygonal ECs, while small capillaries and venules consisting mainly of individual cylindrical ECs through which the blood cells pass in a single file. Moreover, large and small vessels differ in specific markers [73,76] (Fig. 1.5).

The endothelium is composed of a glycocalyx – a thin non-cellular layer that includes glycoproteins, glycolipids, and proteoglycans - and an endothelial cell layer (ESL) – a much thicker zone with chemical and physical properties different from the free-flowing plasma with which it contacts [58]. Both the glycocalyx and the ESL are in the frontline

of blood-tissue interaction and are, consequently, involved in diverse pathophysiological mechanisms. These include, among others, mechanical stress on blood cells (caused by ESL exclusion of red cells and not allowing for significant axial flow) and blood cell/endothelium interaction and inflammation (due to the endothelial influence on the presentation or accessibility of specific adhesion molecules) [58]. Conversely, the thickness, composition, and integrity of the ESL may be altered by several physiological, pathological, and therapeutic processes, such as oxidized low-density lipoproteins, growth factors, hypoxia, ischemia-reperfusion, changes in plasma composition, or enzymes degrading glycocalyx or ESL components [58].

1.3.1. Nitric Oxide Production and Regulation

In normal conditions, the endothelium produces NO and prostacyclin, in response to physical stimuli, hormones, and platelet-derived substances which, in turn, results in vascular relaxation and platelet function inhibition [60]. NO is, in fact, an important element in endothelial function, namely in vascular tone modulation, WBC-adhesion regulation, vascular smooth muscle proliferation, and platelet aggregation [77].

Therefore, NO impacts homeostatic regulation of essential cardiovascular functions, through inhibition of platelet aggregation and adhesion, inhibition of leukocyte adhesion and vascular inflammation, control of vascular smooth muscle proliferation, stimulation of angiogenesis, and activation of progenitor cells (reviewed in [78]). Hence any functional impairment of the endothelium (endothelial dysfunction), may assume clinical significance.

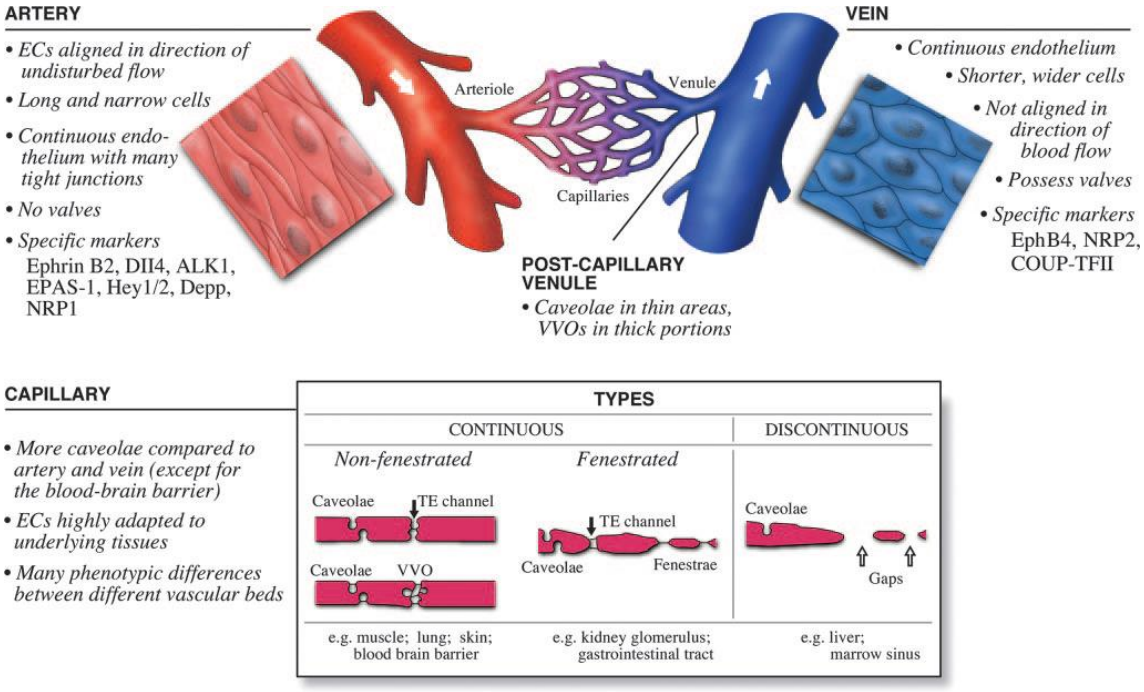


Figure 1.5. ***Endothelial cells (ECs) in arteries, veins, and capillaries.*** Selected phenotypic differences between ECs in arteries, veins, postcapillary venules, and capillaries. ALK: activating-receptor-like kinase 1; Depp: decidual protein induced by progesterone; Dll4: Delta-like 4; EPAS-1: endothelial PAS domain protein 1; NRP1: neuropilin 1; TE: transendothelial; VVOs: vesiculo-vacuolar organelles. Reproduced from Aird, 2007 [79], by permission of Wolters Kluwer Health, Inc.

Once produced it diffuses to vascular smooth muscle cells, where it activates guanylate cyclase which, in turn, leads to increased production of cyclic guanosine monophosphate (cGMP), reduction of intracellular calcium levels, and, ultimately, induces vasodilation [80,81]. NO also reacts with oxygenated Hb to produce methemoglobin (metHb) and nitrate, and with deoxygenated Hb to produce iron-nitrosyl-hemoglobin [82]. This leads to NO-scavenging in conditions where high levels of cell-free Hb are present (e.g., hemolytic states) and, ultimately to decreased NO bioavailability and endothelial dysfunction.

NOS may occur as one of three isoforms [neuronal NOS (nNOS, NOS1), inducible NOS (iNOS, NOS2), and endothelial NOS (eNOS, NOS3)] with different tissue locations and types of expression [reviewed in [78]]. Endothelial NOS (eNOS, or NOS3) is constitutively expressed by ECs, hence is fundamental for the regulation of endothelial NO bioavailability, but has also been detected in other cells such cardiac myocytes, platelets, neurons in the brain, syncytiotrophoblasts of human placenta, and LLC-PK_1 kidney tubular epithelial cells [83]. It is upregulated by calmodulin, through Ca^{2+} mediation [84], heat-shock protein 90 (hsp90), estrogen, vascular endothelial growth factor (VEGF), bradykinin, fluid shear stress [85–87].

On the other hand, it may be down-regulated by caveolin-1 (produced by the caveolae of ECs) [88] (Fig. 1.6). The physiological functions of NO, produced by NOS3, include vasodilation and inhibition of platelet aggregation and adhesion [89,90], inhibition of leukocyte adhesion and vascular inflammation [91,92], control of vascular smooth muscle proliferation [93,94], stimulation of angiogenesis [92,95], and activation of endothelial progenitor cells [96,97].

In enhanced oxidative stress states, like the ones that occur in cardiovascular diseases, increased NO degradation occurs due to its reaction with superoxide anion (O_2^-)[78]. Oxidative states can also convert eNOS from an enzyme that synthesizes NO to an O_2^--producing enzyme – a condition called NOS uncoupling [98]. Other mechanisms that lead to eNOS uncoupling include oxidation of BH4 (a critical eNOS co-factor) [99], L-arginine depletion [100], accumulation of endogenous methylarginines [101], and S-glutathionylation of eNOS [102,103].

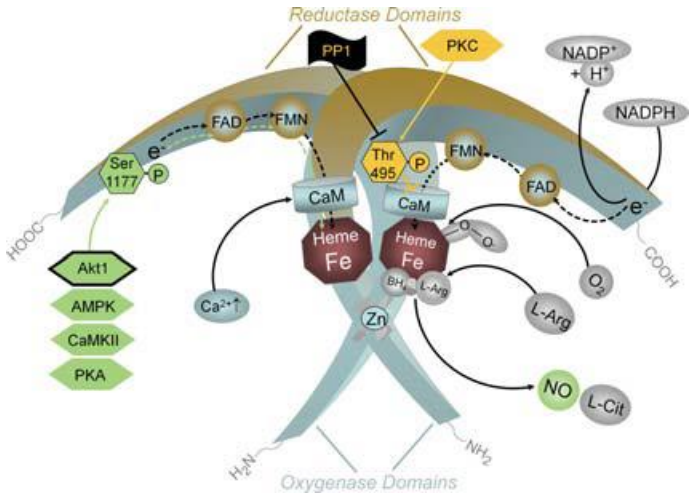


Figure 1.6. ***Regulation of endothelial NOS (eNOS) activity by Ca2+ and phosphorylation.*** Increased intracellular Ca^{2+} leads to an enhanced calmodulin (CaM) binding, which in turn displaces an auto-inhibitory loop and facilitates the flow of electrons from NADPH in the reductase domain to the heme in the oxygenase domain. Ser1177 and Thr495 are established functionally important phosphorylation sites in human eNOS. Exposure to estrogens, vascular endothelial growth factor (VEGF), insulin, bradykinin, or fluid shear stress induces phosphorylation, in an otherwise (resting) unphosphorylated Ser1177. Kinases responsible for phosphorylation (green hexagons) are dependent on the primary stimulus. Estrogen and VEGF induce Ser1177 phosphorylation by activation of serine/threonine kinase Akt, which regulates eNOS function in vivo (framed green hexagon). Akt and the AMP-activated protein kinase (AMPK) may be activated by insulin, Ca^{2+}/calmodulin dependent protein kinase II (CaMKII) mediates the bradykinin-induced phosphorylation of Ser1177, and shear stress leads to phosphorylation of eNOS mainly via protein kinase A (PKA). When the Ser1177 residue is phosphorylated, the electrons' flux increases through the reductase domain, and consequently, enzyme activity also increases. The human eNOS Thr495 residue tends to be constitutively phosphorylated in ECs, and functions as a negative regulatory site. Its phosphorylation is associated with both decreased electron flux and enzyme activity. Protein kinase C (PKC, yellow hexagon) is most probably the constitutively active kinase that phosphorylates eNOS Thr495, and thus reduces eNOS activity (yellow block arrow). On the other hand, dephosphorylation of Thr495 may occur by the action of the protein phosphatase1 (PP1, black flag with black block arrow). Reproduced from Förstermann and Sessa, Nitric oxide synthases: regulation and function, European Heart Journal, 2012, vol. 33, 829-837, [78], by permission from Oxford University Press.

1.3.1. Vascular Cell Adhesion Molecule 1 – Activation and Signaling

Cell adhesion molecules (CAMs) are at the core of cell-cell interactions that take place inside the blood vessels. Their main functions include i) enabling vascular smooth muscle cells to maintain the necessary proximity for cell-cell communication [104], ii) allowing ECs to maintain a highly restrictive barrier preventing excess fluid and protein interstitial loss [105], and iii) support binding of circulating blood cells to the vascular wall, thus promoting processes like inflammation and thrombogenesis [106].

CAMs expressed on the surface of the endothelial layer include selectins (E-selectin, P-selectin, L-selectin), and members of the immunoglobulin gene superfamily (ICAM-1, ICAM-2, VCAM-1, PECAM-1, and MAdCAM-1) which interact with ligands, the integrins, that are expressed on the surface of immune cells or, in the case of SCA, of abnormal RBCs. CAMs mediate not only normal physiological processes but also cellular interactions that contribute to vascular dysfunction and tissue injury which occur in vascular disease states.

VCAM-1 (MIM#192225) is a 100kDa sialoglycoprotein and a highly conserved transmembrane molecule that functions as an adhesion receptor and a signal transducer for leukocytes expressing $\alpha_4\beta_7$ and $\alpha_4\beta_1$ integrin (see below) [107,108]. Besides EC, it is expressed by a variety of tissues, from stromal bone marrow to skeletal muscle [107,109,110]. VCAM-1 exhibits low to negligible expression on unstimulated ECs, but may be strongly upregulated after cytokine [TNF-α, IL-1β, lipopolysaccharide (LPS)] stimulation, hence is closely associated with a pro-inflammatory context [111]. This CAM has long been considered an important signaling molecule, especially in the context of disease. Given its primary role as a receptor for circulating lymphoid, myeloid, and neoplastic cells, VCAM-1 is involved in the pathophysiology of several conditions, such

as atherosclerosis, autoimmune disease, stroke, allograft rejection, asthma, experimental allergic encephalomyelitis, and lymphoid neoplasia [112–118]. VCAM-1 mediates leukocyte binding to ECs through interaction with its integrin counterreceptor, $\alpha_4\beta_1$ integrin (ITGA4, VLA-4 or CD49d/CD29) [119] and by activating endothelial nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to catalyze the release of low levels of reactive oxygen species (ROS) (an activity required for VCAM-1–dependent lymphocyte transmigration through the vascular wall) [120,121]. VCAM-1 is expressed constitutively in lymphoid dendritic cells and stromal fibroblasts of the bone marrow [122,123]. In lymphoid tissues, the interaction between maturing B-cells and dendritic cells at the germinal center is mediated by VCAM-1, a process required for hematopoietic cell maturation. Moreover, it also intervenes in the interaction between the VLA-4-expressing hematopoietic cells and the stromal cells in the bone marrow [122,123]. There are three VCAM-1 isoforms, a complete one with seven domains, and two truncated forms with domains 1-3 or with domains 5-7 [108,119]. Binding with ITGA4 occurs through domains 1 and 4 (Fig. 1.7).

In addition to cytokine and LPS response, several oxidants and bacterial toxins may also trigger transcription-dependent VCAM-1 synthesis. *VCAM1* expression reaches high levels between 2h and 3h after initial exposure and gradually decreases over several days [111]. Molecular activation of VCAM-1 expression is profoundly dependent on two adjacent nuclear factor-κB (NF-κB) and two GATA transcription factor binding sites identified in its gene promoter region [124]. These sites are sensitive to TNF-α, and NF-κB are so in a cell-specific manner, which explains why VCAM-1 expression is constitutive in some cell types and cytokine-dependent in others [107,124,125]. GATA-binding 1 (GATA-1), E-twenty-six transformation-specific 1 (ETS-1), and octamer-binding (Oct) transcription factor binding sites, critical for development, have also been

identified in the *VCAM1* promoter, which is in line with the molecules' role in development (e.g., in skeletal muscle differentiation) [110]. Interestingly, the Oct binding sites have a silencing effect, however, they not only lose activity upon cell differentiation but are also overcome by TNF-α activation of the NF-κB sites [107,126]. VCAM-1 functions in conjunction with other CAMs during chronic inflammation and tumor metastasis [127,128].

ITGA4 (MIM*192975) is an alpha-4 chain of the alpha-beta heterodimeric receptor very-late antigen-4 ($\alpha_4\beta_1$ or VLA-4), a member of the VLA (or very late activation) antigens subset of integrins. VLAs are expressed by nearly all lymphoid and myeloid cells, except for granulocytes and RBCs, and are usually involved in cell-ECM adhesion. ITGA4 is an atypical VLA in the sense that it does not only mediate cell-ECM adhesive interactions (acting as a receptor for fibronectin produced by the ECM) but also as an intercellular adhesion receptor (binding to its ligand VCAM-1). This integrin has been targeted in pharmacological studies given its role in several diseases, like multiple sclerosis (due to its expression at the surface of activated lymphocytes and monocytes, involved in autoimmune response) [129], or in sickle cell disease (namely due to mediating adhesion of young reticulocytes and sickle red cells to the endothelium) [130].

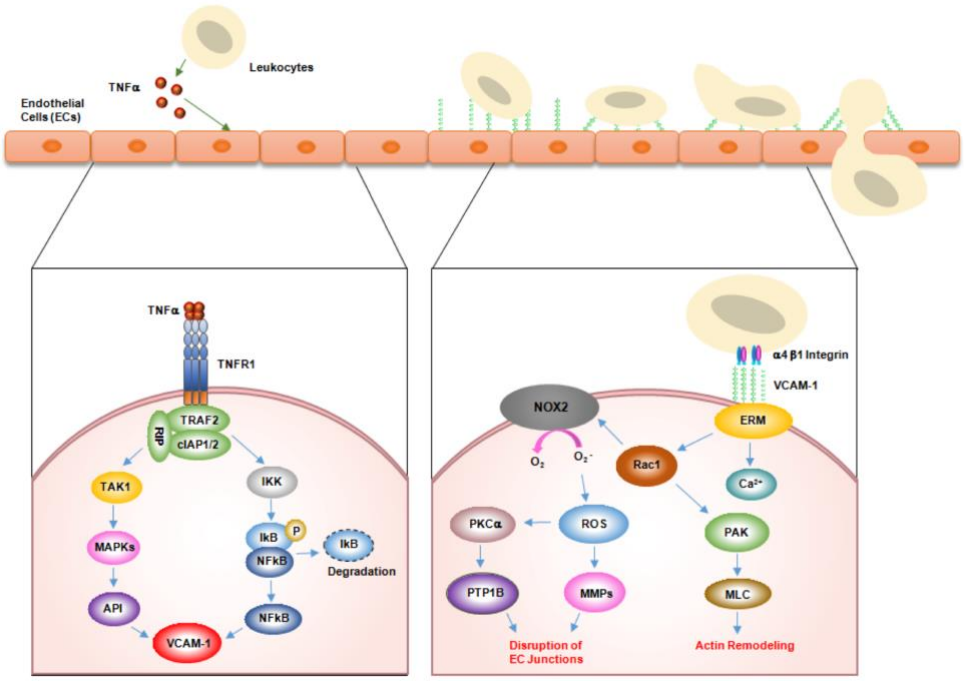


Figure 1.7. ***Mechanism of VCAM-1-mediated leukocyte adhesion and transendothelial migration across the endothelium.*** *Reproduced from Kong et al.* [128]*, with permission of MDPI under a CC-BY-4.0 license.*

1.4. Sickle Cell Anemia

As stated above, SCD is the most common hemoglobinopathy worldwide. More than just a Hb abnormality disorder, the marked clinical heterogeneity renders it as a disease spectrum, with manifestations ranging from very mild to extremely severe and life-threatening (e.g. stroke, pulmonary hypertension, acute chest syndrome). As with phenotypes, genotypes are also varied. From the (most frequent) homozygous $\beta^S\beta^S$ – causing SCA – to compound heterozygosity of β^S with a β allele carrying another mutation (e.g., $\beta^S\beta^C$, $\beta^S\beta^0$, or $\beta^S\beta^+$), several SCD-causing genetic changes are

possible. SCA is not only the most frequent but also the one where the SCD pathophysiological mechanisms occur at the highest degree of severity.

From the first description by James Herrick, in 1910, of abnormally crescent and sickle-shaped red cells on the blood smear of a 20-year-old Grenadan student [131], to the current developments in genome editing [132–136] an astonishing amount of research has been undertaken in SCD. The first finding was later recognized to result from the presence of defective Hb molecules within RBCs, which was the basis for considering this a molecular disease [21]. Subsequently, the actual mechanism was found – the Hb variant S (HbS), produced as a result of an A to T point mutation (HBBc:20A>T, or β^S allele), that leads to a single amino acid substitution of glutamic acid by valine (Glu6Val) in the sixth position of the mutant protein which, in turn, alters RBCs conformation [22] (Figs. 1.8 and 1.9). Since then, molecular mechanisms considered as part of the SCD genetic etiology were further refined. The term SCD is currently applied to a group of hemoglobinopathies caused by the presence of the β^S allele, in homozygosity (SCA), or compound heterozygosity with another mutant *HBB* allele that either changes the β-globin chain or reduces *HBB* expression (Fig. 1.9). Substitution of the same glutamic acid residue by lysine (Glu6Lys) gives rise to HbC, which when inherited concomitantly with HbS results in HbSC disease, a less severe form than SCA. HbS/β-thal disease is yet another form of SCD, arising from the coinheritance of β-thalassemia (β^0-thal or β^+-thal) and HbS. Although SCA is the most common and severe form, the compound heterozygote genotypes also lead to the production of sufficient HbS to promote intracellular sickling [137]. In those individuals, the majority of Hb is HbS, whereas in individuals with the sickle cell trait (heterozygotes for HbA and HbS) HbA is the main Hb form present (Fig. 1.9).

HbS has a low O_2 affinity hence, in hypoxic conditions, it polymerizes inside RBCs, a determinant pathobiological event in SCD [138], that culminates in the abnormal (crescent or sickle) RBC shape (Fig. 1.8), and altered microrheological as well as biomechanical properties, that impact their aggregability, deformability, and cell adhesion [33]. Those changes play a significant role in the pathophysiological events occurring both in small and large vessels that ultimately contribute to end-organ damage. Hence SCD, and specifically SCA, can be considered a vascular disease with complex specific pathways according to the affected organ [139].

Even though the central causal event in SCA is a homozygous point mutation, its marked clinical heterogeneity mimics a multifactorial mode of transmission. Health-related quality of life and life expectancy also show high variability [140–142]. This overall heterogeneity is thought to occur as a consequence of modifier factors, including environmental, socio-demographic, as well as genetic modulators, which has complicated the adoption of overall effective therapeutic strategies. The knowledge of the mechanisms underlying vasculopathy in SCA is fundamental for the identification of potential therapeutic targets, namely for drug development. Additionally, it would address the need for pharmacological, patient-specific, treatment alternatives voiced by SCD patients worldwide, as reported in the recent *Sickle Cell World Assessment Survey* (SWAY) [143]. The following sections provide an overview of the main aspects of SCA, from epidemiology and disease burden to therapeutic approaches. Specific emphasis is given to pediatric cerebral vasculopathy, given its considerable impact on SCA management in children.

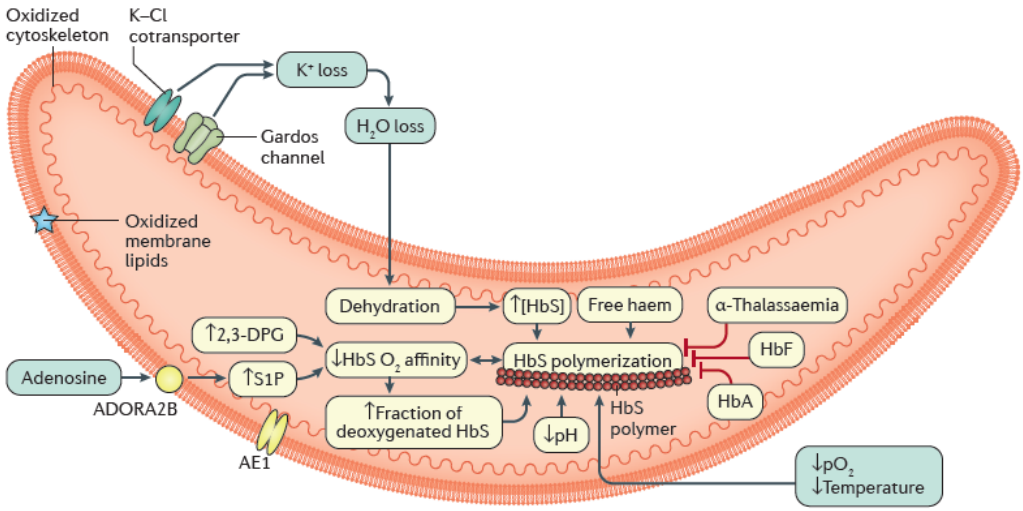


Figure 1.8. ***Red blood cell alterations underlying sickling.*** HbS polymerization damages the red blood cell (RBC) membrane and causes: 1) release of lipid-rich microparticles which results in cells with an increased mean corpuscular HbS concentration, which further favors polymerization; 2) altered cation transport; 3) epitope exposure that promotes cell adhesion and facilitated premature cell destruction in the reticuloendothelial system and intravascularly. RBC life span is thus reduced from the normal 120 days to 20 days, and consequently, hemolytic anemia is present, regardless of the occurrence of vaso-occlusive events. Factors like HbS concentration, partial pressure of O_2 (pO_2), pH, temperature, and 2,3-phosphoglycerate (2,3-DPG) concentration, affect the HbS polymerization. Increased 2,3-DPG and sphingosine-1-phosphate (S1P) lead to diminished HbS-O_2 affinity, and consequently higher levels of deoxyHbS, favoring polymerization further. Cationic imbalance (e.g., potassium, K+, loss through the Gardos channel), leads to RBC dehydration, which further increases HbS intracellular concentration. ADORA2B: adenosine receptor A2B; AE1: band 3 anion transport protein; HbA: hemoglobin A; HbF: fetal hemoglobin. Reprinted by permission from Springer Nature: Nature, Nature Reviews Disease Primers, Sickle Cell Disease, [142], Copyright 2018.

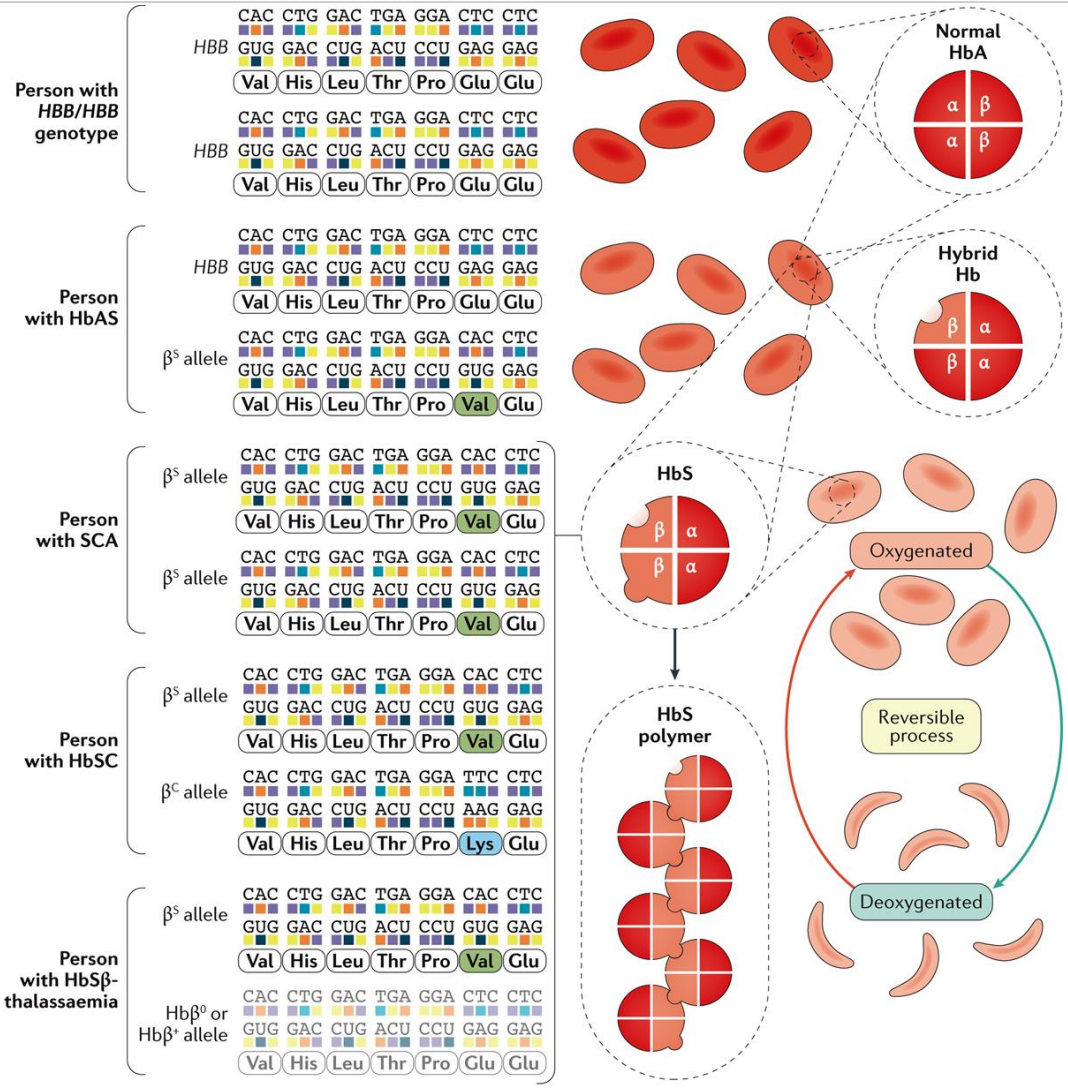


Figure 1.9. ***Genetic mechanisms in sickle cell disease and red blood cell shape change.*** The β^S allele results from an A to T mutation in the *HBB* gene. The subsequent glutamic acid to valine substitution in the 6th position of the mature β-globin chain leads to hemoglobin S (HbS) production. When both *HBB* alleles are mutated and at least one of them is a β^S allele sickle cell disease (SCD) arises. In low oxygen conditions, HbS forms polymers that distort red blood cells (RBCs) into a sickle shape. The presence of one β^S allele leads to the benign sickle cell trait condition (HbAS) but not SCD, while homozygosity for the β^S allele results in the most severe SCD form, sickle cell anemia (SCA). Other common SCD genotypes include HbSC and HbSβ-thal. Patients with HbSC have a β^S allele and another mutated β allele (β^C allele, resulting from a Glu6Lys mutation) that causes the production of hemoglobin C (HbC). The concomitant occurrence of the β^S allele and a β-thalassemia mutation (β^0 or β+ alleles) that eliminates or decreases β-chain production, leads to HbSβ-thalassemia. HbSβ^0-thalassemia is very similar to SCA, except for the presence of microcytosis (abnormally small RBCs) HbSβ^+-thalassemia is usually milder than SCA, due to low-level expression of HbA.

1.4.1. Epidemiology and Global Burden

SCD affects about 70% of the 300,000-500,000 children born each year with hemoglobinopathies (Fig. 1.10). The public health implications of the disease are significant and even though an increasing proportion of affected children now survive past five years of age, the risk of premature death remains especially in underdeveloped countries [144,145].

It is believed that the β^S mutation arose in a multicentric fashion, originating independently at least four times in Africa and one in Asia [146]. The different origin sites were associated with the five main β-globin cluster haplotypes and these were named after the geographical locations where they were first reported: Bantu (or Central Africa Republica, CAR), Benin, Senegal, Cameroon, and Arab-Indian [146].

SCA is particularly common among people of Sub-Saharan Africa, India, Saudi Arabia, or Mediterranean descent. High frequencies of the sickle-cell trait (heterozygosity for β^S mutation, HbAS, fig. 1.8) seem population-specific and at-risk individuals may be numerous in certain population groups and geographic areas. Those correspond to areas currently (or formerly) endemic for malaria due to an evolutionary link between heterozygous state and protection against *Plasmodium falciparum* infection. However, with the massive migration events occurring worldwide the number of carriers and affected individuals may be high even in non-endemic areas, increasing the global public health burden [144]. In these geographic regions, clinical diagnosis may not be sufficient for prognosis and management since several factors, including genetic, environmental, and social, seem to influence disease severity.

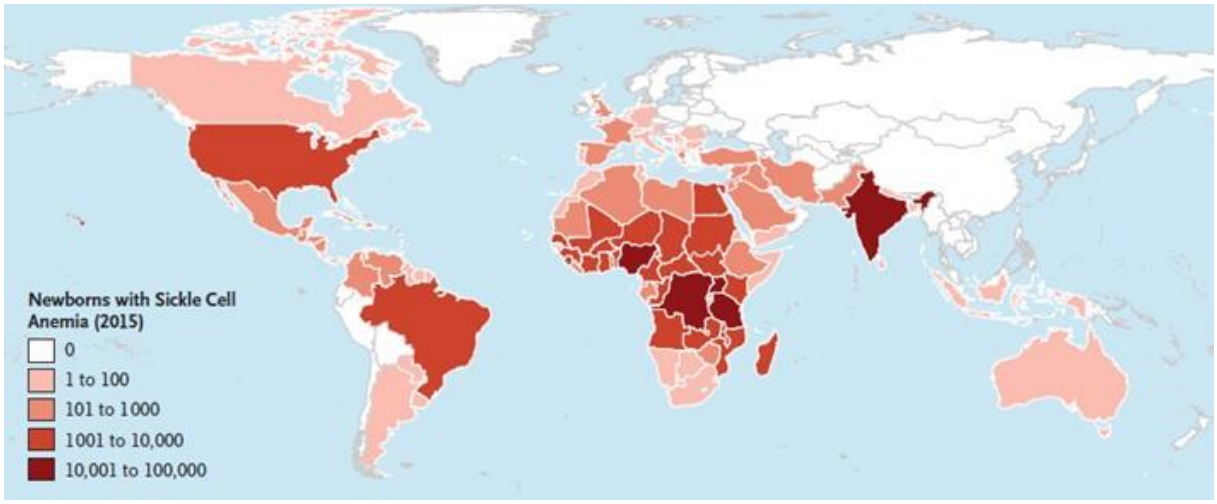


Figure 1.10. ***Number of newborns with sickle cell anemia in each country in 2015.***
Reproduced with permission from Piel *et al.* [147], Copyright Massachusetts Medical Society.

In Portugal, the current incidence and carrier prevalence are unknown due to the lack of a national registry and country-wide screening. Previous studies identified a heterogeneous distribution across the country, ranging from virtually zero in the north to about 1.1% in the south along with high prevalence (5%-6%) "pockets" in the regions of Coruche, Alcácer do Sal, and Pias [148,149] (Fig. 1.11). These high prevalence "pockets" seem to correspond to areas where malaria was formerly endemic, which is in favor of a positive selection of the mutation [148,150]. Genetic migration of the sickle cell trait into the autochthonous Portuguese population is thought to have occurred in two distinct waves: the first probably from the Mediterranean basin, between the 8th and 13th centuries (Benin haplotype); and the second with the slave trade, around the 15th century (Bantu and Senegal haplotypes) [148]. Slaves were brought to work on the swampy rice fields in the low valleys of the rivers Sado, Guadiana, and Tejo, where malaria was endemic [150].

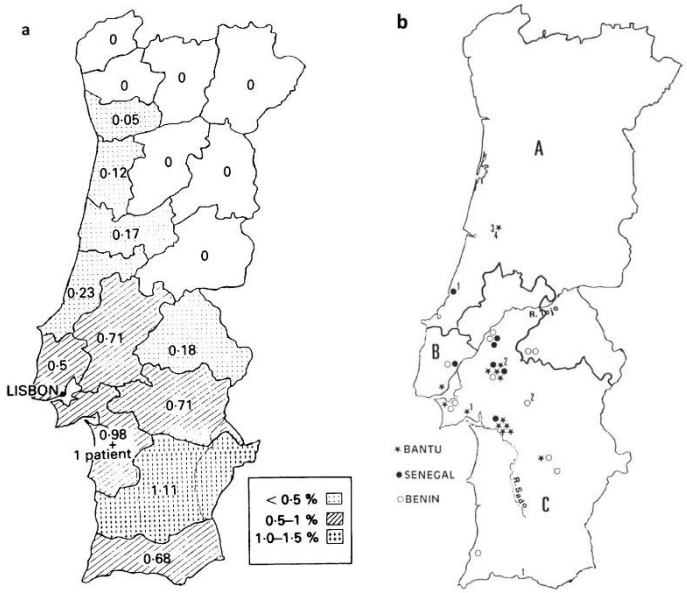


Figure 1.11. ***Prevalence and distribution of (a) carriers of hemoglobin S trait in Portugal (%) and (b) HBB haplotypes of sickle cell disease in Portugal****. a) Reproduced and adapted from* [150] *by permission of the BMJ Publishing Group Limited; b) Reproduced from* [148]*, with permission from J. Lavinha.*

The previously mentioned main African haplotypes (Bantu, Benin, and Senegal) are found in phenotypically Caucasian Portuguese (Fig. 1.11) which may be related to the widespread areas of Portuguese settlement in Africa [148,150]. More recent migration (in the 1980s) of significant numbers of people originating from the Portuguese-speaking African countries (PALOPs) and Brazil is most certainly associated with an increase in HbS carrier prevalence, especially in host regions (Inez et al., 1993) like the Greater Lisbon area.

1.4.2. Overview of Sickle Cell Anemia Pathophysiology

The pathophysiology of SCD has been reviewed extensively [139,142,147,151–153]. All patients with SCA have the same homozygous c.20A>T (or Glu6Val) missense mutation in the

HBB gene and produce the same biochemical phenotype – the presence of HbS. Nevertheless as mentioned previously phenotypic heterogeneity is considerable, with the involvement of multiple organs and different degrees of disease severity. Patients may have a combination of complications and differ from each other in disease severity, age of onset, rate of progression, and ultimately in health-related quality of life. Discrepancies between patients in high- and low-income settings are also apparent [140–142].

As stated previously, the insoluble HbS ($\alpha_2\beta^S_2$) polymerizes into long rigid fibers, under hypoxic conditions, with each fiber consisting of seven intertwined double strands with cross-linking, that distort and damage the membrane and cytoskeleton of RBCs [33]. Consequently, RBCs become sickle-shaped with altered microrheological and biomechanical properties, that impact their aggregability, deformability, and cell adhesion [33]. The shape changes are initially reversible, occurring in cycles of oxygenation and deoxygenation (oxy-deoxy). However, as the number of oxy-deoxy cycles increases, the altered RBCs become irreversibly sickled (SSRBCs), a state associated with increased fragility, shorter life span, and higher propensity to adhere to the endothelial wall[154]. Being more fragile, SSRBCs rupture easily (hemolysis) which subsequently results in anemia. The shorter life span (one week contrasting with 120 days for normal RBCs) stimulates increased production and excess of circulating immature RBCs, or reticulocytes (also called stress reticulocytes). Compromised deformability, which impairs their normal flow through capillaries, contributes to blood vessel blocking or vaso-occlusion. Vaso-occlusion is a multifaceted process that may result from complex interactions between endothelial cells, neutrophils, and SSRBCs. A multistep multicellular model has been proposed, despite the complexity and still incomplete knowledge of the exact mechanisms involved. That model is based on four

steps: endothelial activation, recruitment of adherent leukocytes, interactions of sickle RBCs with adherent neutrophils, and vascular obstruction by heterotypic multicellular aggregates [155]. Endothelial cells are activated as a response to internal and external pro-inflammatory stimuli (e.g., TNF-α, IL-1β, and IFN-γ), followed by recruitment of circulating neutrophils, especially in the endothelium of post-capillary venules [54,156–160]. The neutrophils then adhere to circulating adhesive SSRBCs through macrophage-1 antigen (Mac-1), thus obstructing venular blood flow (transiently or prolongedly). This decreases RBC transit time, causes tissue ischemia distally to the obstruction, enhances RBC sickling, leads to further neutrophil recruitment and heterotypic cell-cell aggregation. The overall process culminates in vaso-occlusion, cumulative organ damage, and ultimately in life-threatening crises [155]. In this sense, SCD vaso-occlusion is similar to ischemia-reperfusion injury and vascular inflammation described in several cardiovascular diseases.

Inflammation, chronic hemolysis (especially intravascular hemolysis), with the release of Hb and heme and subsequent decrease in NO bioavailability, as well as enhanced oxidative stress, strongly contribute to vascular pathology or vasculopathy. NO reduction shifts the vascular balance towards vasoconstriction (Fig. 1.12), while the activated endothelium expresses several CAMs (e.g., VCAM-1, ICAM, selectins), and undergoes cell proliferation thus contributing also to the vaso-occlusive process [161].

Overall these processes work in a self-sustained cycle of repeated cell activation, cell adhesion, inflammation, hemolysis, oxidative stress, vaso-occlusion, and ischemia-reperfusion injury, ultimately lead to vasculopathy.

1.4.2.1. Chronic Inflammation

Several endothelial functions are susceptible to changes or impairment which could lead to cell and tissue injury. Inflammatory alterations are one cause of endothelial dysfunction and have been described in the pathogenesis of cerebrovascular disease [115,162].

Intracellular HbS polymerization in RBCs is increased under hypoxic (or acidic) conditions, and higher levels of polymerized HbS enhance RBC sickling. The cell membrane damage resulting therefrom causes exposure of cell membrane elements, like PS, and the production of reactive oxygen species (ROS) [163]. SSRBCs may cause endothelial injury which, in addition to intravascular hemolysis, activates ECs promoting the expression of pro-inflammatory signals like endothelin-1 or the NF-κB pathway. Activation of NF-κB signaling triggers the upregulation of CAMs, such as E-selectin, VCAM-1, and ICAM-1 [164,165], which mediate leukocyte recruitment and adhesion [166]. The increased adhesive properties of SSRBCs together with CAM overexpression, contribute to amplified adhesive interactions between SSRBCs and ECs, but also with leukocytes and platelets. This stimulates a pan-cellular activation that culminates in the upregulation of cytokines, such as TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-1 (IL-1), IL-3, IL-6, IL-8, and macrophage colony-stimulating factor [156,167–169]. Additionally, high serum levels of the inflammatory modulators prostaglandins E1 and E2, as well as of the angiogenic growth factor have been found in SCD.

The damaged membrane of SSRBCs makes them prone to premature cell death (see 1.4.2.2) which results in the release of Hb and its prosthetic moiety, heme, into the plasma. The cell-free Hb contributes to inflammation in SCD by scavenging NO, promoting oxidative stress, triggering apoptosis, and endothelial barrier

dysfunction [170–172]. In addition to upregulating endothelial CAM expression, heme may also lead to activation of circulating neutrophils and formation of neutrophil extracellular traps in the vasculature and subsequent organ injury [157]. Furthermore, studies in murine SCD models indicate that cell-free heme/hemin pro-inflammatory signaling is dependent of toll-like receptor-4 (TLR4) [157,158,173].

1.4.2.2. Intravascular Hemolysis

Together with inflammation, hemolytic anemia is also a chronic SCD complication and more severe in SCA patients than in any of the other SCD forms. Hb concentration varies not only across SCD genotypes but also among individuals with the same genotype. In SCA, the red cell survival range is 2 to 21 days, a variability also exhibited in clinical markers of hemolysis – total Hb concentration, reticulocyte count, bilirubin level, and lactate dehydrogenase (LDH) levels [174–178].

Hemolysis results from sickle RBCs' fragility. It can occur inside or outside the blood vessels (intra- or extravascular hemolysis, respectively). Chronic intravascular hemolysis results in the release of Hb, heme, and other RBC cellular components (microparticles) into the circulation. This strongly impacts NO bioavailability and is a major underlying mechanism of several SCD complications. Cell-free Hb (oxyHb, Fe^{2+}) binds to NO, rapidly converts it into bio-inactive nitrate (NO_3), shortening its half-life, and reducing its ability to diffuse across cell membranes. MetHb (Fe^{3+}) is also produced in this process [151,171]. Also, there is an arginase release that depletes L-arginine, an amino acid critical for NO production. The subsequent decrease in NO bioavailability will lead to vascular tone imbalance, platelet activation and aggregation, as well as transcriptional up-regulation of VCAM-1, ICAM-1, P-selectin, and E-selectin [179]. Free Hb, heme, and heme iron catalyze the production of oxygen radicals further

decreasing NO endothelial availability and promoting endothelial dysfunction [180]. The vascular tone is also disturbed, with the balance being skewed towards vasoconstriction, endothelial activation, and proliferation [151].

The relationship between hemolysis and stroke in SCD has been indirect. Stroke was previously associated with lower Hb concentration [181], while co-inheritance of α-thal was shown to have a protective effect in SCA patients with stroke [182]. The presence of α-thal was also demonstrated to be significantly higher in children with normal transcranial Doppler (TCD) cerebral blood flow velocities when compared with children with high TCD flow velocities [183]. The latter is a strong risk factor for pediatric stroke in SCD (*see section 1.4.4.3*). Moreover, the fact that the current therapeutic management strategies include chronic blood transfusions, which reduce hemolytic rate, plasma Hb level, and also stroke risk, further argues in favor of a link between hemolysis and stroke risk [184].

Two distinct SCD subphenotypes have been proposed based on hemolysis rate. The first (hemolysis–endothelial dysfunction) was centered around the association between high hemolytic rate and predisposition for vasculopathy-related complications, namely stroke, pulmonary hypertension, leg ulceration, and priapism [151,171,179,185]. The second subphenotype (viscosity–vaso-occlusion) was identified in individuals with high rates of vaso-occlusive painful episodes and is associated with acute chest syndrome and osteonecrosis [151]. However, this two-subphenotype model has not been consensual, and validation has proved challenging. More importantly, the model did not address retinopathy (leading to loss of sight) or renal vasculopathy (prior to kidney damage), two important SCD manifestations that are associated with vasculopathy [186].

1.4.2.3. Endothelial Activation and Dysfunction

The balance between endothelial injury and recovery strongly influences endothelial function. The distinction between endothelial activation and dysfunction is oftentimes missing and both concepts are frequently mixed. Since the endothelium is an “active” (as opposed to “quiescent”) tissue, constantly reacting to maintain vascular homeostasis, activation may not necessarily be detrimental. Conversely, endothelial dysfunction is always a pathological condition [187], that occurs when the normal functions of the endothelium are shifted towards reduced vasodilation, a pro-inflammatory state, and pro-thrombotic properties [60]. Cytokines like IL-1β, IL-6, or TNF-α activate the endothelium and may lead to decreased NO synthesis and *VCAM1* overexpression [109,168,188–191]. On the other hand, replacement of injured ECs may occur after the production and release of circulating endothelial progenitor cells from the bone marrow [188]. Patients with cardiovascular disease (e.g., coronary heart disease, atherosclerosis) and its risk factors (e.g., hypertension, diabetes, obesity) exhibit endothelial dysfunction, namely reduced ability to produce NO and NO-mediated vasodilation [66,179,192].

Changes in the expression of surface adhesion molecules, leading to adhesion to SSRBCs and other blood cell types, promote multicellular aggregation, further contributing to vascular occlusion. Increased “adhesiveness” towards endothelial cells is also apparent, especially in the microvasculature – an interaction that causes “arrest” of those aggregates inside vessels, and contributes to the proliferation of the endothelial layer and increased blood viscosity [193]. Furthermore, the overexpression of adhesion molecules by the endothelium also results in shedding to the vascular lumen. High levels of soluble CAMs, like VCAM-1, constitute strong biomarkers of endothelial activation and further contribute to progressive activation and proliferation. Ultimately

this results in vaso-occlusion and further intra- and extravascular hemolysis [165,193–195]. Adhesion molecules that are overexpressed by sickle red cells (both reticulocytes and erythrocytes), such as VLA-4, CD36, and basal cell adhesion molecule/Lutheran protein (BCAM/Lu) work as mediators of the adhesion between these cells and the microvasculature walls [196]. For example, as mentioned previously, VLA-4 binds to VCAM-1, expressed by the activated ECs, and to fibronectin produced by the ECM. On the other hand, CD36 acts as a linker between thrombospondin (TSP) and von Willebrand factor (vWF) and the endothelial vitronectin receptor or exposed heparin sulfate proteoglycans [196]. The increased number of circulating immature RBCs in affected individuals [196] with their overexpression of adhesion molecules strongly contributes to the overall higher adhesiveness of erythroid cells. Additionally, sickle reticulocytes show increased gene expression subunits of the VLA-4 (CD49d and CD29) and also of CD36, when compared with reticulocytes of control individuals [197]. The sickle red cells are also dense and dehydrated and this results in an exposure of negatively charged phosphatidylserine on their plasma membrane, a factor that can activate the coagulation cascade, and indirectly, further inflammation [198]. SSRBCs-EC adhesion also promotes changes in the microvasculature hemodynamics, reducing blood flow, promoting conditions for HbS polymerization, and favoring the retention of SSRBC and multicellular aggregates, which ultimately results in vaso-occlusion [199].

1.4.2.4. Ischemia-Reperfusion Injury

The vaso-occlusion promoting events also contribute to the pathogenesis of ischemia-reperfusion (I/R) injury, namely to microvascular dysfunction. Ischemia-reperfusion injury in SCA differentiates it from other chronic anemia states [200]. The resulting

obstructed blood flow results in ischemic alterations in downstream tissues, exposing all ECs in the post-ischemic tissues to the same damaging effects. When the obstruction is resolved, an inflammatory reaction causes local injury that may progress to systemic inflammation, damaging organs distal to the injury site, and potentially ending in life-threatening multi-organ injury or failure [201,202]. Studies in sickle murine models have demonstrated the importance of I/R injury [203,204], while some aspects are inferred from other models. In general, notwithstanding the common I/R insult, arterioles, capillaries, and venules react in a site-specific manner. Acetylcholine endothelium-dependent vasodilation response is diminished in arterioles [205,206]. On the other hand, the endothelial barrier function is impaired in capillaries, resulting in interstitial edema, and leukocyte capillary plugging reduces the number of perfused capillaries, which enhances tissue hypoxia [207]. Nevertheless, reperfused post-capillary venules are the vessels that bear the stronger impact of vascular response to ischemia-reperfusion, especially through increased leukocyte-endothelial cell adhesion, platelet-leukocyte aggregation, excessive albumin extravasation, and higher oxidant production [208,209]. In SCD, vaso-occlusion precipitates the initial cell damage and causes local tissue hypoxia. Unable to undergo aerobic respiration, the oxygen-deprived cells become deficient in ATP, and after mitochondrial dysfunction associated with intracellular hypercalcemia, they swell and die [210]. Several mechanisms, like immune system and platelet activation, as well as aggregation [201] and activation of the NF-κB pathway [211], follow cell death and are relevant to SCD [212,213]. Monocyte chemoattractant protein-1, VEGF, and platelet-activating factor are other mediators of adhesion, inflammation, and coagulation relevant to I/R injury in SCD [189,212,214–216]. Microvascular dysfunction due to I/R injury in SCD includes changes like microvascular permeability, pro-inflammatory and pro-coagulable endothelial cell activation,

alterations in vasoactive mediator levels, and production of ROS [200]. Hypoxia causes necrosis, leading to increased levels of hypoxanthine, and also to conversion of xanthine dehydrogenase into xanthine oxidase. Upon normalization of blood flow and oxygen delivery, those levels of hypoxanthine and xanthine oxidase become toxic [203,217,218]. NO deficiency also occurs as a result of inflammatory, hemolytic, and oxidant processes (*see sections 1.4.2.1, 1.4.2.2, and 1.4.2.5*). Loss of NO signaling promotes activation of leukocytes, platelets, and NF-κB, while also contributing to the release of P-selectin and vWF from Weibel-Palade bodies [219].

Recurrent microvascular occlusions lead to persistent vascular damage and all organs are potentially affected by systemic I/R injury. However, animal studies indicate that different organs show different degrees of susceptibility to that damage, with the brain, heart, and kidney being more vulnerable to local ischemia and I/R injury [204].

1.4.2.5. Oxidative Stress

Oxidative stress in SCA results from the participation of several players – sickle RBCs, leukocytes, monocytes, and vascular endothelium – in the altered redox biology of SCA. SSRBCs have high baseline concentrations of ROS, hydroxyl radical (OH^-), $O_2^{\cdot}$, and hydrogen peroxide (H_2O_2), when compared to normal RBCs which suggests the presence of a pro-oxidant environment in SCA patients even before the onset of clinical manifestations [220]. Oxidant mediators may also take part in ischemia-reperfusion injury and inflammation which are frequent in SCA (*see sections 1.4.2.1 and 1.4.2.4*).

HbS quickly oxidizes inside SSRBCs, to form metHb, hemin (ferric iron, Fe^{3+}), and superoxide. As a result, several RBC activities are impacted, such as cytoskeletal oxidation, membrane lipid peroxidation, and phosphatidylserine exposure. The

glycolytic pathway that SSRBCs use to produce energy, due to lack of mitochondria, produces NADH and allows to reduce metHbS to the more innocuous ferrous state. However, SSRBCs also show an upregulation of NADPH oxidase, which leads to a decrease in NADH. SSRBCs also show a depletion in glutathione, an additional scavenger of ROS, thus enhancing oxidative stress in these cells [221]. Sickling and RBC oxidative damage lead to RBC shedding microparticles which, in turn, have been shown to induce ROS in cultured ECs and cause vaso-occlusion in sickle mouse kidneys [222]. This process is potentially mediated by heme bound to externalized microparticle phosphatidylserine [223].

The constant supply of cell-free Hb and heme/hemin provided by chronic hemolysis described earlier (section 1.4.2.2) also promotes a pro-oxidant vascular environment. Besides the interactions between oxyHb with NO, it may also react with H_2O_2 through the Fenton reaction to form hydroxyl free radical ($OH^{\cdot}$) and metHb. The latter may further degrade into hemin, which is a major RBC damage-associated molecular pattern (DAMP). The hemolysis-related ROS contribute to the activation of inflammation and adhesion in endothelial cells, platelets, and neutrophils. This may ultimately result in vaso-occlusion, as demonstrated by the study of Ghosh et al [224], where direct hemin infusion induced acute VOC in sickle mouse lungs, which was improved by inhibiting P-selectin-mediated pathways.

The oxidative damage effects are further enhanced in SCD due to failure/downregulation of several antioxidant pathways or oxidant scavenger molecules. Hemolysis-related rapid clearing of haptoglobin and hemopexin, which scavenge cell-free Hb and hemin, respectively [225] is one example. Co-administration of haptoglobin and hemopexin was shown to restore microvascular blood flow in sickle mice, that presented with acute vascular stasis due to infusion of haptoglobin

or hemin [158]. Furthermore, upregulated gene expression of hemopexin in hemin-infused Townes-SS mice (SCA murine model) prevents microvascular occlusion, increases expression of the protective liver nuclear factor erythroid 2-related factor 2 (Nrf2) and heme-oxygenase-1 activity, as well as decreasing pro-inflammatory nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). On the other hand, venostasis is worsened in sickle cell hemopexin knock-out mice exposed to hemin [226]. Nrf2 activation has an antioxidant protective effect in SCD, by removing hemolysis-derived free heme [227], and it has also been shown to induce HbF by binding to γ-globin antioxidant response element [228].

Heme oxygenase-1 (HO-1) is the inducible isoform of the enzyme heme oxygenase (HO), and its gene *HMOX1* expression is increased by the presence of heme, which the enzyme converts to biliverdin, CO, and ferrous iron, along with several additional protective effects. Belcher and colleagues have shown that, in the Townes-SS murine model, a single co-infusion of hemopexin and haptoglobin upregulated HO-1 expression and lowered NF-κB activity in several tissues, and also protected from vaso-occlusion for up to 48h [229]. The authors also suggested that the benefits of haptoglobin and hemopexin do not depend exclusively on rapid Hb and hemin clearance from circulation, since the effect of HO-1 on NF-κB activity and venostasis were independent of Hb and hemin plasma concentrations. In a subsequent study using the same murine model the administration of oral CO upregulated Nrf2 and HO-1, downregulated NF-κB activity, soluble VCAM-1, and venostasis [230].

Other mechanisms that may further contribute to increased oxidative stress in SCD include high mobility group box 1 (HMGB1)-mediated TLR4 signaling that (activates NF-κB pathway and) may affect endothelial CAMs expression (e.g., P-selectin), and mitochondrial dysfunction in platelets, in addition to non-heme dependent

mechanisms [224,231,232]. The latter include NADPH oxidase, xanthine oxidase (XO), and uncoupled eNOS, all of which generate oxygen free radicals, thus promoting endothelial dysfunction. Myeloperoxidase (MPO) generated by activated neutrophils also produces oxidants that scavenge NO, further contributing to endothelial dysfunction [233,234]. Increased levels of the oxidative stress biomarkers MPO, HO·, lipid peroxidation, and total thiols have recently been confirmed in SCD, while antioxidants superoxide dismutase (SOD), glutathione, and catalase levels were reduced [234,235].

1.4.2.6. Sickle Cell Anemia Vasculopathy

Being mainly a chronic vascular disease, the majority of SCD pathological mechanisms arise due to disturbance of the homeostasis inside the micro and macrovasculature. Although the core mechanism results from altered properties of sickle cells and their interactions with other blood cells or the vascular endothelial wall, several other mechanisms lead to the SCD phenotype (Fig. 1.12).

Vasculopathy in SCD arises as a combined multistep process that comprises, but is not limited to, decreased NO bioavailability, oxidative stress, ischemia-reperfusion injury, elevated leukocyte count, platelet activation, and increased levels of multiple inflammatory mediators [236]. Therefore, vasculopathy is central to several of the clinical complications as it often occurs before end-organ dysfunction. SCD-related vasculopathies include, among others: moyamoya (that often precedes cerebral infarcts/hemorrhage), proliferative retinopathy (prior to eyesight loss), pulmonary vasculopathy (associated with pulmonary hypertension), and renal vasculopathy (preceding chronic renal disease)[186].

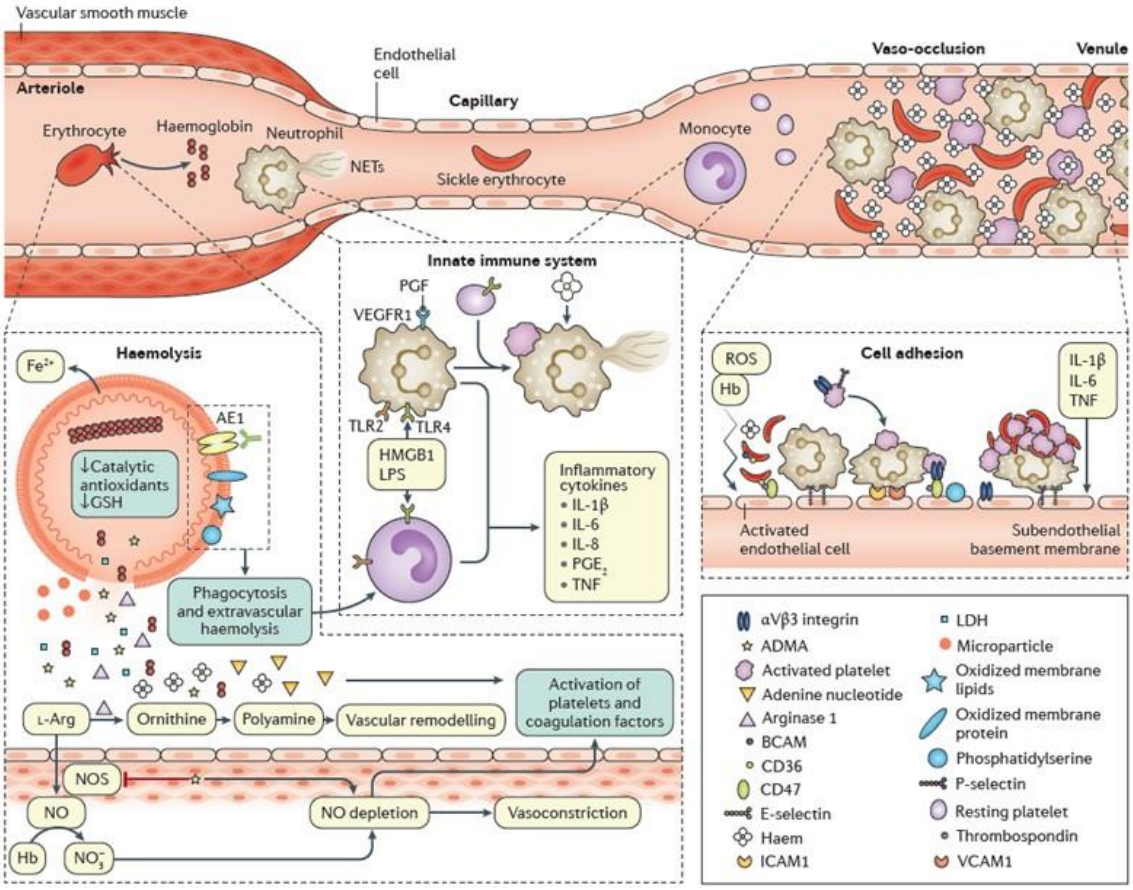


Figure. 1.12. ***Pathobiology of sickle cell disease.*** An interplay between several mechanisms particularly important in arterioles, capillaries, and post-capillary venules, from hemolysis to endothelial activation/dysfunction in a feedforward manner and culminating in vaso-occlusion. The damage and dysfunction caused by HbS on the RBC membrane result in hemolysis. Membrane proteins that are oxidized expose antigens that bind to existing antibodies and membranes extrude phosphatidylserine. These mechanisms favor RBC phagocytosis by macrophages, and ultimately extravascular hemolysis. RBC content is released into the plasma through intravascular hemolysis, leading to NO scavenging by cell-free Hb, arginase 1 depletion of the L-arginine substrate of NOS, and asymmetric dimethylarginine (ADMA) NOS inhibition. NO is further depleted by reactive oxygen species (ROS), triggering vasoconstriction and vascular remodeling, particularly in the lung. NO and adenine nucleotides deficiency cause activation of platelets and blood clotting proteins. Activation of the innate immune system also occurs, due to cell-free heme and other damage-associated molecular pattern (DAMP) molecules. Monocytes and macrophages are activated to release inflammatory cytokines by ligand-bound TLR4 and TLR2, thus ensuing an inflammatory state and activation of ECs. Platelets' TLR4 activation favors their adhesion to neutrophils, which in turn release DNA to

form neutrophil extracellular traps (NETs). Adhesion of circulating blood cells to each other and to the activated endothelium contributes and may even promote vaso-occlusion. Activated ECs that express P-selectin and E-selectin, in postcapillary venules, can bind rolling neutrophils. Activated platelets and adhesive sickle RBCs may adhere to circulating or EC-bound neutrophils, forming multicellular aggregates. Sickle RBCs may also bind directly to the activated endothelium. Only a few examples of the complex and redundant receptor-ligand interactions involved in the adhesion of circulating cells to the damaged endothelium and exposed subendothelium are represented. AE1: band 3 anion transport protein; BCAM: basal cell adhesion molecule; GSH: glutathione; HMGB1: high mobility group protein B1; ICAM1: intercellular adhesion molecule 1; LDH: lactate dehydrogenase; LPS: lipopolysaccharide; PGE2: prostaglandin E2; PGF: placenta growth factor; TNF: tumor necrosis factor; VCAM1: vascular cell adhesion protein 1; VEGFR1: vascular endothelial growth factor receptor 1.

Cerebral vasculopathy is a severe complication of SCA, especially in children, with stroke constituting a highly devastating manifestation [181]. As with other manifestations of SCA, genetics may have a vital role in determining phenotypic heterogeneity. The interpatient variability in SCA has fuelled research aimed at identifying genetic modifiers of disease severity which would be potential targets for vasculopathy treatments (*see section 1.4.5*).

1.4.3. Pediatric Clinical Presentation

Despite the marked clinical heterogeneity, chronic hemolytic anemia, painful crisis episodes, and widespread organ damage are common in SCA patients. While some patients may have an almost normal life, others develop crises from an early age and may die in infancy or as young adults. Even though there have been marked improvements in life expectancy and supportive care in high-income countries, high

childhood mortality and morbidity at a young age are still observed in low-income countries. Lack of nation-wide newborn screening programs in most of these countries hampers the timely diagnosis of children which may ultimately lead to failure in access to specific care.

The more frequent pediatric clinical manifestations include acute pain crises (also called vaso-occlusive crises, VOCs), splenic sequestration, recurrent infections due to functional asplenia, acute chest syndrome, cerebrovascular accidents, cholelithiasis, nocturnal enuresis, hematuria, reduced (and delayed) growth [237]. Crises may be vaso-occlusive (painful or visceral), aplastic or hemolytic and organ damage may be severe. VOCs have a variable clinical presentation, presenting in early childhood with dactylitis (swollen, tender, or erythematous hands or feet), with the highest incidence between 6-12 months of age [238]. The frequency, intensity, duration, and location of pain crises may vary considerably [238,239]. Cerebrovascular accidents, namely stroke, are the most impactful events in pediatric SCA. High levels of HbF as well as co-inheritance of α-thalassemia have been associated with milder SCD phenotypes, and therefore have been considered major disease modifiers [152]. However, this modulation only accounts for a small portion of the phenotypical heterogeneity observed.

The risk of infection from encapsulated organisms is higher in children with SCD due to functional asplenia. Given the spleen damage caused by SSRBCs splenic dysfunction occurs in the early years of life (and, consequently, functional asplenia), and invasive bacterial infections (namely *Streptococcus pneumoniae* and *Salmonella sp.*), or parvovirus B19 are frequent. Meningitis, respiratory virus infections (e.g., influenza), as well as malaria, may also affect these children. Penicillin prophylaxis, vaccines, and antibiotic treatment have decreased infection-related mortality in high-resource countries. Parvovirus B19 infections are either mostly without complications

or may be severe, associated with aplastic crises, and require blood transfusions to restore reticulocyte count [240]. On the other hand, in endemic regions, malaria is associated with a high mortality rate in children [241]. The infection susceptibility of children with SCD also poses additional risk in the recent COVID-19 pandemics.

Acute chest syndrome (ACS) is an acute pulmonary process that occurs exclusively in SCD patients. It manifests as a new pulmonary infiltrate that involves at least one complete lung segment (on chest X-Ray) and is associated with respiratory symptoms (cough, wheezing, tachypnea), chest pain, and fever [237]. The clinical course of ACS varies from mild illness to respiratory failure, and even death, with children having a lower mortality risk than adults [242].

Pediatric neurological disease risk is high in SCD and may be devastating, particularly in the case of overt stroke (*a detailed discussion is provided in section 1.4.4*).

The chronic hemolysis in SCD may also cause bilirubin gallstones and lead to biliary tract disease. Furthermore, if the RBC sickling in the hepatic sinusoids is significant, the child may develop intrahepatic cholestasis and consequently extreme conjugated hyperbilirubinemia, abdominal pain, hepatomegaly, coagulopathy, and elevated transaminases. This can cause liver failure and even death [237].

Renal damage is also frequent in children with SCD. Due to infarction in the renal medulla, they tend to produce diluted urine (hyposthenuria), which causes polyuria (increased urinary frequency) and nocturnal enuresis (persistence of urination in bed at night).

Finally, growth patterns may also be impaired in children with SCD, not as a result of the genetic mutation but probably due to chronic severe anemia, suboptimal nutrition, hypermetabolism, and potential endocrine dysfunction [237].

1.4.4. Pediatric Cerebrovascular Disease in Sickle Cell Anemia

1.4.4.1. Impact and Risk Factors

The first report on the natural history of stroke in SCD was from Powars and colleagues, in 1978, who described a peak incidence in infants and recurrence in 67% of untreated patients. The highest risk was observed in children within the first 3 years after the initial event [243]. This original study was followed by others that aimed either to assess incidence and prevalence – like the *Cooperative Study of Sickle Cell Disease* (CSSD) [181] – or assess stroke screening and/or prevention strategies – like the pivotal *Stroke Prevention Trial in Sickle Cell Anemia* (STOP) trial [184]. SCA is the most common cause of pediatric stroke, as children with SCA have a 300-fold increase in stroke risk. Moreover, stroke is more frequent in SCA than in other SCD presentations [181]. Subclinical cerebral infarctions (silent cerebral infarctions, SCI) only apparent on magnetic resonance imaging (MRI) are even more common.

These lesions correlate with marked neuropsychological deficits, and approximately half of the children with SCIs may require life-long support or custodial care [244]. Even after following the recommended therapy, children who experience a stroke may suffer significant physical and/or cognitive deficits that are limiting to their quality of life [245]. As with other SCD complications, cerebrovascular manifestations are varied, ranging from extensive, large vessel distribution infarcts to more subtle lacunar infarcts, while differing in terms of epidemiology, clinical features, and pathology [246].

The risk of cerebrovascular complications is highly increased in SCA, predisposing patients to ischemic or primary hemorrhagic stroke [181,243,247] (Fig. 1.14). Pediatric stroke and SCIs occur at a high cumulative rate in SCA [181,248].

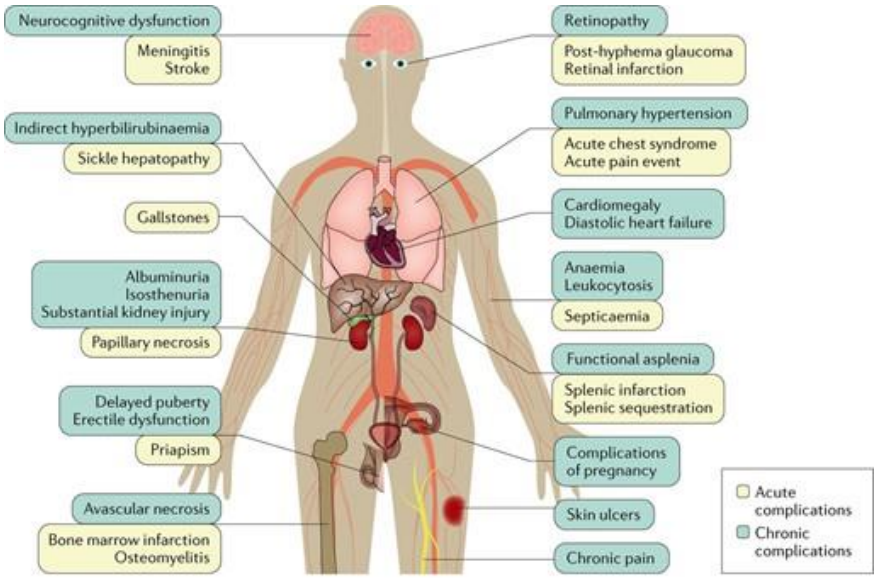


Figure 1.13. ***Common clinical complications of sickle cell disease.*** Acute (yellow boxes) and chronic complications (blue boxes) are depicted. Acute complications usually require urgent medical care, especially acute pain crises. Chronic complications arise as the patient with SCD ages and usually result in organ damage and may contribute to premature death. In pregnancy, the most common complications include pre-eclampsia, intrauterine growth restriction, preterm delivery, and perinatal mortality. Reprinted by permission from Springer Nature: Nature, Nature Reviews Disease Primers, Sickle Cell Disease [142], Copyright 2018.

A 20-year-old SCA patient has an 11% probability of having already experienced at least one stroke event, and this risk increases to 24% by age 45 years [181]. Whereas ischemic stroke is the more common form in young children, intracranial hemorrhage tends to occur later in life [181]. The numbers are even higher for SCIs, with about 37% of children suffering at least one event before reaching 14 years of age. Furthermore, children that have had previous SCIs are at increased risk of developing stroke.

Different SCD populations seem to present with different stroke rates. While in the CSSD trial an 11% cumulative incidence of stroke and a 17% prevalence of unsuspected cerebral infarction on MRI were found, the French Study Group on SCD

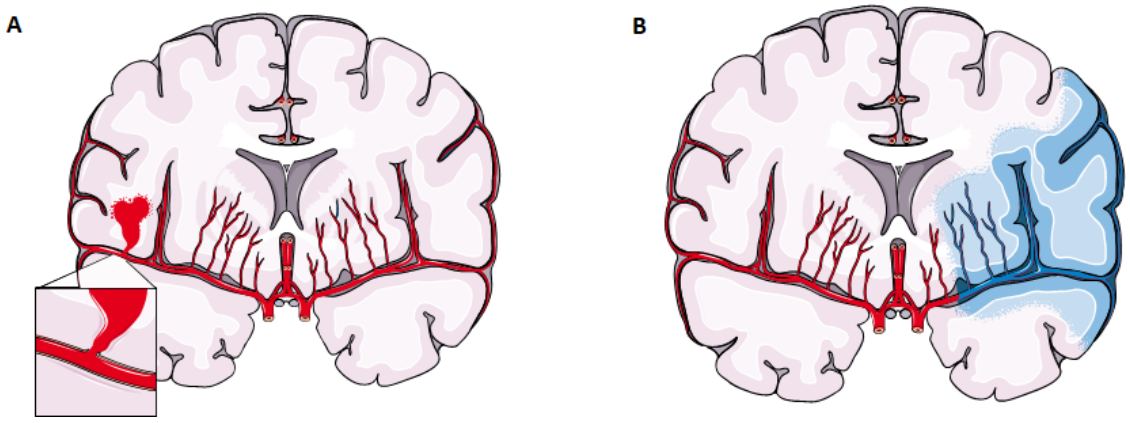


Figure 1.14. ***Forms of overt stroke.*** *(**A**)* ***Hemorrhagic stroke****. A ruptured vessel leads to brain hemorrhage in the area surrounding the affected vessel; (**B**)* ***Ischemic stroke****. Vaso-occlusion leads to ischemia "downstream" of the blockage site. Created using* ServierMedArt*, under a CC- BY-3.0 license.*

reported a 6.7% prevalence of clinically overt stroke and a 15% prevalence of SCIs on SCA patients of primarily African descent [249]. A slightly lower stroke rate was also found in a Jamaican birth cohort – 7.8% by age 14 years [250]. In other populations, pediatric stroke is rare, like in Arabian children [251] and SCI has also a lower prevalence in Kuwaitian children with SCD [252]. These differences are important when devising prevention, screening, diagnosis, and therapeutic strategies in different populations. Moreover, this will also potentially affect those strategies in admixed populations, if ancestry is not taken into account.

Brain imaging is fundamental for confirming overt strokes (clinically apparent and with abrupt onset of neurological manifestations) and diagnosing SCIs (present on brain MRI, but without clinical symptoms or external signs that correlate to the neuroimaging findings) [248].

1.4.4.2. Pathophysiology of Pediatric Cerebrovascular Manifestations

Cerebral vasculopathy has been identified as a major underlying mechanism of ischemic stroke, based on cerebral angiography or magnetic resonance angiography (MRA) [253–255]. Concomitantly, the most common histopathological finding in SCD-related cerebral vasculopathy is endothelial damage in the mid to large-sized brain arteries [256,257]. That damage occurs in particular at the branch points, and infarcts in SCA patients occur more frequently where the stenosis and occlusion especially at the area at the bifurcation between the internal carotid artery and the middle cerebral artery – the Circle of Willis (Fig. 1.15). It was also found that the endothelial damage usually leads to intimal layer proliferation, fibrin deposition, and thrombus formation [186] (Fig. 1.16). The arterial stenosis and occlusion may result in the formation of a pattern of collateral formation – moyamoya [258].

The distribution of infarctions observed on those pathological examinations is consistent with the hypothesis that overt strokes in SCD are a consequence of mid- to large-vessel disease, affecting mainly the distal internal carotid, proximal middle cerebral, and anterior cerebral arteries [259,260]. Nevertheless, there is still a lack of knowledge about the mechanisms that determine cerebral vasculopathy progression.

The lower oxygen affinity of HbS and its polymerization under hypoxic conditions trigger a cascade of events from chronic hemolysis, to microvessel occlusion, endothelial activation and dysfunction, inflammation, and ischemia/reperfusion injury.

Tissue ischemia in the brain results in ischemic stroke, which in turn tends to occur in the border zones, or watershed regions, even in the absence of large-vessel vasculopathy [261–263].

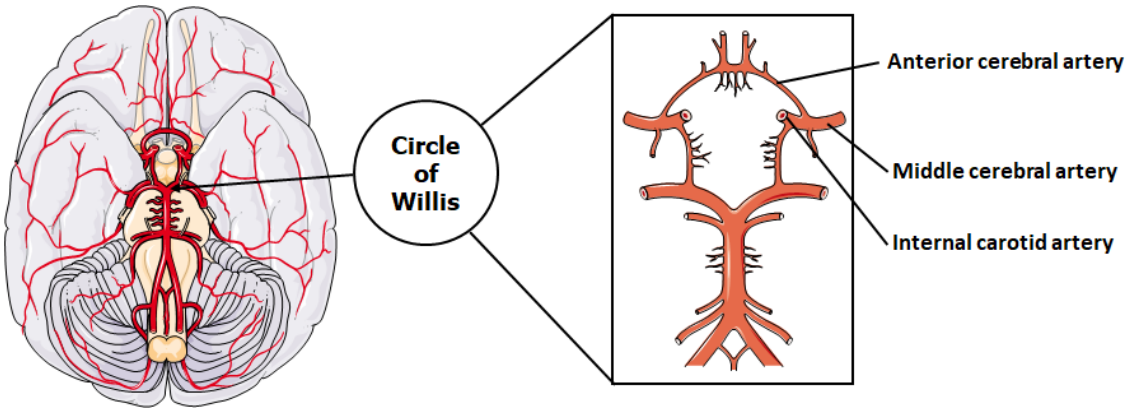


Figure 1.15. ***Brain arteries.*** The main posterior arteries of the Circle of Willis are emphasized. Created using ServierMedArt, under a CC-BY-3.0 license.

In non-SCD ischemic stroke patients arteriolar dilation occurs to maintain cerebral blood flow (CBF), and increases in oxygen extraction fraction (OEF, the fraction of oxygen that is extracted from blood by the brain tissue), contribute to keep the cerebral metabolic rate of oxygen utilization ($CMRO_2$) under conditions of decreasing perfusion pressure [264,265]. When these compensatory mechanisms are insufficient to meet the tissue metabolic demands, lowering the $CMRO_2$, the result is a cerebral infarct [266]. In those adult patients that have carotid occlusive disease, elevated hemispheric may be a robust indicator of stroke risk [267]. CBF is elevated in children with SCD [263,268], a compensatory mechanism for chronically low arterial oxygen content (oxygen-carrying capacity) due to anemia. A recent study proposed that elevated OEF in the deep white matter reflects a metabolic stressed cerebral tissue and is associated with increased stroke risk in those children [263]. Cerebral regional changes in CBF, OEF, and $CMRO_2$ were observed in the deep white matter of pediatric SCD patients, with OEF peaking when CBF and $CMRO_2$ are at their minimum. This was consistent with failure to meet metabolic demand in regions of lower CBF and $CMRO_2$, despite high OEF, as these

were areas with higher infarct density. The overlap between areas of high infarct density and high OEF, but not with low CBF, indicated that high OEF would be a stronger indicator of stroke risk, than low CBF [263]. Furthermore, a subsequent study showed that cerebral metabolic stress may be ameliorated with hydroxyurea therapy in children with SCD [269] (*see section 1.4.4.4*). That strategy could be an adjuvant to the most used imaging tool for stroke risk prediction – the transcranial Doppler ultrasonography for measuring the time-averaged mean of maximum velocity (TAMMV) in the middle cerebral artery. That quantitative assessment of stroke risk proposed by the pivotal STOP trial is based on Bernouilli's principle of fluid dynamics (*see section 1.4.4.3*). Briefly, this principle states that the speed of a fluid increases when there is a decrease in pressure. In the case of cerebral vasculopathy that decrease takes place distally to the region where a partial occlusion (or narrowing) of the blood vessel occurs. Hence, if one measures the blood flow velocity in the large arteries mentioned earlier, this could be a strong indicator of the occurrence of a vaso-occlusive or stenotic event.

Children with SCA have a high prevalence of SCIs and these are the most frequent form of SCA-related neurological injury. They may appear very early in life and remain undetected unless the child undergoes brain-imaging exams or until an overt stroke occurs. As mentioned previously, SCI risk is cumulative up to 37% of children with SCA have suffered an SCI by age 14 years and this increases up to 53% in adults by the age of 32 years [181,270,271]. In addition to brain injury, SCI events are associated with increased overt stroke risk [254], failure to meet academic milestones (Schatz et al, 2001), and increased cognitive impairment [245,249,272].

Contrary to strokes, SCIs do not seem to result from cerebral macrovasculopathy. Initially, it was suggested that they were spatially circumscribed to the white matter of

the frontal and parietal lobes [254]. Subsequent studies demonstrated the occurrence of white matter loss [273] and disrupted matter integrity [274] as well as border zone [273,275] and cortical, wedge-like infarcts [262]. Border zone infarcts are suggestive that ischemic mechanisms may result from global reduction in arterial oxygen content, cerebral hemodynamic factors, or both, while wedge-like infarcts indicate a predominance of thromboembolic factors [276].

With no apparent external manifestations, SCI diagnosis relies heavily on brain imaging technology, like MRI. A large prospective study with a pediatric SCA cohort showed that cerebral blood flow decreased as infarct density increased, thus confirming that SCIs predominantly occur in the border zone vascular distribution within the white matter of the frontal and parietal lobes [276]. Also, the results demonstrated that 90% of children had SCIs within a relatively small border zone region, measuring 5.6% of total brain volume, and that they occurred in the region of low blood flow.

Lower baseline Hb levels (anemia), higher baseline systolic blood pressure, and male sex are known risk factors for SCA-related SCIs [270,277]. Long-term effects of SCIs include failure to meet academic milestones and impaired cognitive function [244,278].

1.4.4.3. Screening and Diagnosis of Stroke and SCI

Diagnosis and screening of cerebral vasculopathy in SCA were made possible with the technological advances in neuroimaging. The best prognostic tool available for pediatric stroke in SCA is transcranial Doppler ultrasonography (TCD). MRI or MRA are more suited to (confirm stroke and) diagnose SCI and progressive vasculopathy, respectively.

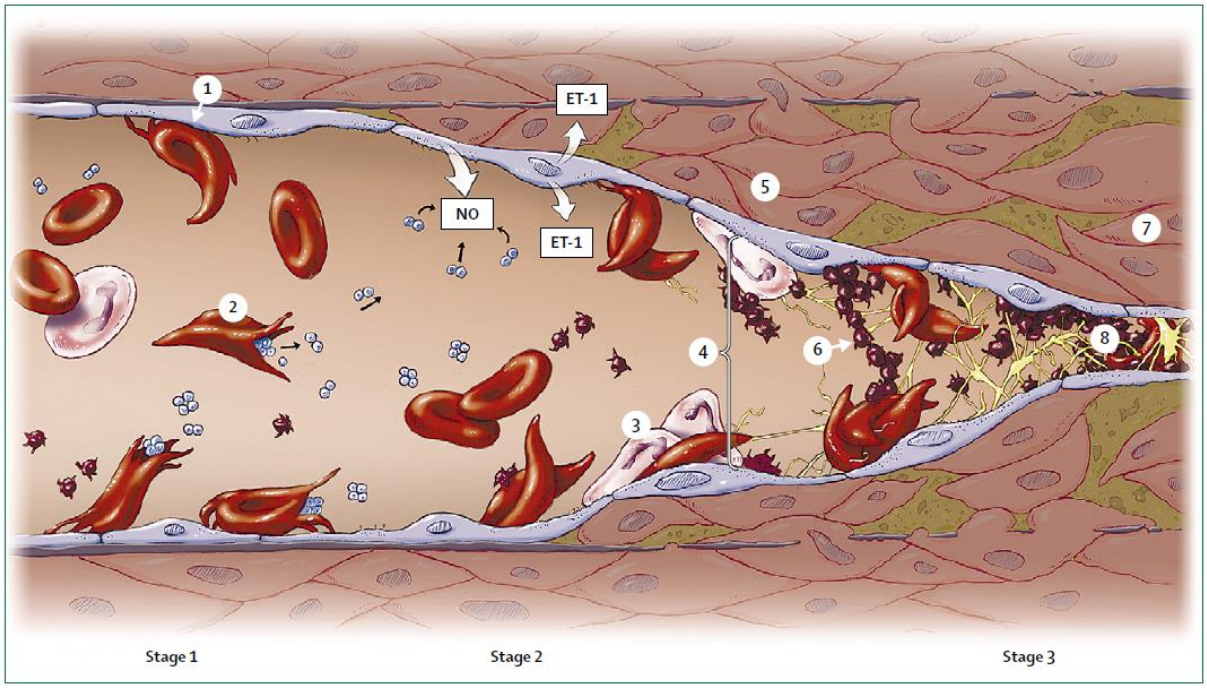


Figure 1.16. ***Vasculopathy and stroke in sickle cell disease.*** Sickle RBCs' abnormal adherence to the endothelium (1) and hemolysis (2) make these cells critical for the development of cerebrovascular disease in sickle cell disease. Both mechanisms lead to a pro-inflammatory state, partly reflected by leukocyte adhesion (3) and platelet aggregation (6). The vasomotor tone is also enhanced (4) due to increased endothelin-1 (ET-1) secretion and nitric oxide (NO) scavenging by cell-free hemoglobin dimers. The vascular lumen becomes narrower as a result of proliferation of smooth-muscle cells and fibroblasts within the intimal layer (5). All these mechanisms ultimately result in vasculopathy (7) and occlusion (8). Reprinted from The Lancet, vol. 5, [161], p. 505, Copyright 2006, with permission from Elsevier.

The TCD screening for stroke risk relies on Bernouilli's principle of fluid mechanics, mentioned earlier, and was the basis of the ground-breaking trial in primary stroke prevention – the STOP trial [184]. Based on the study, an increase in blood velocity in the terminal portion of the internal carotid or the middle cerebral artery, resulting from intracranial vasculopathy would be a strong indicator of future strokes. The TAMMV in the middle cerebral artery, mentioned earlier, was the chosen parameter – measurements <170 cm/s indicate low risk, while velocity values of 170–199 cm/s are

associated with moderate or “conditional” risk. The high stroke risk group included children with TAMMV values ≥200 cm/s. Children with SCA and stroke, detected by TCD, who received at least monthly regular (or chronic) blood transfusion (CBT) had a 92% reduction in the relative risk of overt stroke when compared with the observation group. The STOP trial results allowed for the establishment of a new standard of care that was followed by a marked decrease in the incidence of pediatric stroke in SCA [184]. In the STOP2 trial, patients receiving transfusions for at least 30 months showed normalization of TCD values, whereas children discontinuing transfusions presented with increased values [279].

Subsequently, the *Stroke with Transfusions Changing to Hydroxyurea* (SWiTCH) trial showed that transfusion and chelation were more effective than HU in the treatment of patients with SCD, stroke, and iron overload [280].

Another clinical trial that involved 29 sites in the United States, Canada, the United Kingdom, and France, the *Silent Cerebral Infarct Transfusion* (SIT) trial, aimed primarily at determining if CBT was effective in preventing new ischemic injury in the form of overt stroke or SCI detected on MRI of the brain [281]. In that study, which followed up over three years, 6% of children who were treated with CBT, compared with 14% of non-treated, had ischemic injury events (one stroke and five new or enlarged silent infarcts, in the treated group; seven strokes and seven new or enlarged SCIs, in the observation group). However, no full-scale IQ effect was observed as a result of CBT in the SIT trial [281].

Subsequently, the *TCD With Transfusions Changing to Hydroxyurea* (TWiTCH) trial indicated that HU may be effective for primary stroke prevention in patients with high TAMMVs [282].

Current stroke screening strategies rely on TCD velocities, and stroke risk stratification is strongly based not only on the pivotal STOP trial but also on the subsequent trials described above. Nevertheless, not all patients with high TCD values develop stroke, and children with low measurements may also experience stroke events [283]. Furthermore, the intra-individual variation in cerebral arterial blood flow velocities requires more than one TCD measurement per patient [284], which limits its application in low-resource settings. The optimal duration of CBT treatment is still unknown and even though sustaining HbS levels below 30%, some patients are still at risk for stroke recurrence or TIAs [285,286]. Since CBT does not appear to effectively prevent cerebral vasculopathy [287], some authors have suggested that long-term follow-up should include yearly MRI and MRA [288].

SCI management and prevention are even more challenging. Contrary to overt strokes, the SCI pathophysiology is not associated with macrovasculopathy, and therefore normal to conditional TAMMVs are not indicative of a low risk of SCIs. Moreover, treatment with CBT is insufficient to prevent SCI occurrence and recurrence [289].

The challenges in the existing screening and diagnostic strategies, despite the beneficial role of the current imaging techniques, highlight the need for novel biomarkers with high specificity and sensitivity to design more adequate screening and therapeutic options that are effective in all patients.

1.4.4.4. Treatment Strategies

In general, current SCA treatments target symptom relief and, where possible, primary prevention. Even though an established prevention therapy is in place for pediatric stroke prevention in high-resource settings, the challenges for cerebrovascular disease treatment are common to the ones for other SCA manifestations. Therefore a

discussion of treatment strategies for pediatric cerebrovascular manifestations overlaps with the ones for SCA in general. Obtaining a one-size-fits-all therapeutical solution is highly unlikely due to the complex nature of SCA. While upstream targeted strategies address the β-globin gene cluster expression, namely the Hb switching mechanism, downstream targeted approaches are directed to the multitude of pathophysiological mechanisms of SCD. Upstream approaches are recent and rely on circumventing the genetic defect, whether directly (by correcting the mutation) or indirectly (by reversing the physiologic switch in Hb production). Downstream strategies are especially challenging due to the high complexity of SCD pathophysiology, where much is still unknown.

Management and treatment options have constantly improved in high-resource settings as a result of advances in research and policies, which reflects in higher life expectancy and lower morbidity and mortality rates. Present approaches include newborn screening programs, prophylactic therapies including oral penicillin (for effective reduction of infection rate), and vaccination against the most common infections agents that affect children with SCD (*Pneumococcus*, *Haemophilus influenza,* and *Meningococcus*); analgesia (for pain/VOC crises relief); CBT (described earlier); hydroxyurea (HU); and bone marrow (hematopoietic stem cell) transplant (HSCT) [290–292]. Allogeneic HSCT [293–296] and, more recently, gene therapy approaches – using lentiviral vectors for gene addition [297–299], or genome editing for gene correction [136,300] – aim to cure the disease. HSCT has been applied successfully in pediatric SCD patients at risk for stroke, with an event-free survival rate of 95.3%, [296]. However, this strategy used HLA-matched sibling donors and myeloablative conditioning to improve outcomes and, in general, for full effectiveness HSCT requires adequate donor pools consisting in a representative donor population with a similar

genetic structure to the recipient population [301]. Unrelated donor HSCT has also been approached however, it was not considered sufficiently safe for widespread application due to the risk of graft-versus-host disease [302]. Gene therapy where autologous hematopoietic stem cells are genetically modified would be an attractive alternative to HSCT. Comparatively, the main advantages of gene therapy are that they do not require matched donors, thus avoiding the risk of graft-host disease or graft rejection associated with HSCT, neither require the aggressive conditioning regimen (namely immunosuppression) prior to the procedure [303]. In theory, this would allow performing the infusion of genetically modified cells and patient follow-up in many pediatric and adult hematopoietic cell transplantation centers, without requiring high expertise in allogeneic HSCT. However, in reality, and especially in low-resource settings where basic therapeutic needs are still difficult to be fulfilled, it would be challenging to implement these strategies, due to costs and resource requirements. Comprehensive care programs that include education to healthcare professionals and patient caregivers are also important for SCD health management improvement.

Current treatment specific for pediatric stroke in SCA also suffers from these limitations. The application of CBT and/or HU to high-risk children identified by TCD is now the standard of care in high-resource settings [145]. The goal of CBT is to maintain HbS at less than 30% [304]. Nevertheless, CBT has been found to attenuate but not eliminate the incidence of new cerebral infarcts [281,289], is associated with the risk for alloimmunization and iron overload, requires a robust antigen-matched blood supply, and an overall effective blood banking system. Despite the potential benefits to a large number of patients, transferring those strategies to low-resource settings, namely where SCA is more prevalent (like Sub-Saharan Africa) will require the establishment of partnerships/consortiums, improvements in health policies, and establishment of

comprehensive care programs [305]. Another challenge in SCD treatment is that management of hematological disease alone does not prevent vasculopathy progression in many children [306–308], which further reinforces the postulate of additional genetic risk factors playing a role in cerebral vasculopathy.

Other therapies have been proposed in high-income regions, such as encephaloduroarteriosynangiosis (surgical revascularization through an external bypass procedure), and, more recently, gene therapy [145,186]. All of these require further validation and have been devised as a secondary stroke prevention strategy. Furthermore, and like the approaches mentioned earlier, they are of limited access in regions with fewer resources.

In terms of pharmacological disease-modifying treatments, and despite the numerous recent clinical trials, only four substances have been approved by the European Medicines Agency (EMA) and the Food and Drug Administration (FDA, USA) – HU, L-Glutamine, voxelotor, and crizanlizumab.

HU was the first to be EMA- and FDA -approved, for SCD treatment. This therapeutical agent is believed to act on a variety of mechanisms to ameliorate SCD-related clinical complications. Those mechanisms include erythroid regeneration, NO-related increase in soluble guanylate cyclase activity, and cyclic guanidine monophosphate (cGMP) that stimulate *HBG* expression or NO increase through long-term posttranscriptional rise in eNOS levels [309,310]. The increase in HbF production counteracts the destructive effects of HbS, thus acting as a disease modifier. These effects translate into several beneficial effects in SCD such as a lower incidence of pain episodes and acute chest syndrome, as well as a reduction in the number of hospitalizations [311,312]. Furthermore, HU decreases mortality and morbidity associated with VOC events [313], cerebrovascular accidents [282,314], and proteinuria [315], thus

increasing overall survival in SCD patients [316]. By reducing the number of blood transfusions (and therefore, hospitalizations) needed, and having been shown to have a similar effect to CBT on TCD velocities in SCD children at high risk of stroke, it has been considered essential in pediatric stroke prevention in SCD [282,312]. More recently, HU was demonstrated to scavenge free radicals and induce the expression of antioxidant genes [317]. This indicates a role in counteracting oxidative stress by activating the expression of several genes in the antioxidant pathway.

HU has been used in low- to middle-income countries probably due to its less challenging application when compared to other therapies developed thus far. The application has been done more frequently in children than in adults, as children (<18 years old) show a more robust and higher capacity to reactivate HbF expression. This is beneficial as an early intervention strategy and important long-term SCD treatment [318]. However, laboratory surveillance costs are a challenge in those countries [145]. Recently, the *Realizing Effectiveness Across Continents with Hydroxyurea* (REACH) trial showed beneficial effects of HU and promising results on its potential widespread administration in African children with SCD [319]. Despite the wide use of HU, research on the full spectrum of the specific molecular pathobiological mechanisms of SCD it affects is ongoing. That knowledge will also be beneficial in understanding why some patients do not seem to respond to HU (non-responders).

L-Glutamine and more recently voxelotor are the only drugs that, besides HU, currently have official approval for SCA treatment. L-Glutamine is a NAD precursor which provides a supplementation that will counteract the NAD depletion of sickle red cells [320]. The drug has been described to reduce acute complications of SCD, both in pediatric (<5 years of age) and adult patients, namely VOC painful crises and hospitalizations [320]. Despite rapid approval, much is still unknown regarding the

L-glutamine mode of action. Furthermore, in the study in which FDA approval was based no change in hematologic parameters was demonstrated and it also failed to show improvements in terms of quality of life and well-being [321].

Voxelotor mode of action has been assessed in several studies, like the HOPE clinical trial [322]. It acts as an inhibitor of HbS polymerization and therefore as a potential overall disease modifier. Voxelotor was shown to increase Hb levels, as HU, thus reducing the number of acute anemia episodes. Levels of hemolysis markers, namely bilirubin (and to a lesser extent, reticulocyte and LDH levels) were also decreased. Nevertheless, it was not demonstrated to have a beneficial action in VOC or acute chest syndrome.

Crizanlizumab is a humanized anti-P-selectin antibody that was shown to be effective in reducing VOC frequency [323]. The rationale was to decrease both RBC and leukocyte adhesion to reduce vaso-occlusion however, it did not ameliorate hematologic parameters or quality of life.

The discrepancy between the numerous clinical trials in place and the low number of officially approved pharmacological agents reduces options for treatment of patient-specific clinical conditions. In the absence of curative therapies available to the majority of SCD patients, it is imperative to provide alternatives to improve survival, well-being, and quality of life. This is a critical issue for both patients and caregivers, who in a recent survey reported increasing expectations in the development of therapeutical management specifically focused on the diverse clinical complaints that affect their quality of life [143].

Several ongoing and nearly finished clinical trials may provide other potentially effective and accessible pharmacological options. For this effort to succeed, further research on the different pathobiological mechanisms underlying SCA is essential. As the knowledge of SCA mechanisms improves, namely the ones underlying pediatric

stroke, new possibilities arise to develop therapies. The majority of treatments address the overall disease instead of specific manifestations, regardless of their impact on patient well-being. Past therapeutical efforts have focused primarily on prevention or symptomatic management. Nevertheless, HU, for example, profoundly affects pediatric stroke risk/recurrence and is still widely used in the management of this clinical manifestation.

The most promising target mechanisms for pharmacotherapy identified so far, besides HbF stimulating agents, include adhesion, reperfusion injury, coagulation, and hemolysis [130,323–329]. Adhesion targeting drugs being tested include selectin inhibitors (e.g., rivipansel) [324] and heparinoids [329]. On the other hand, downregulation of inflammatory pathways is the objective of drugs like regadenoson, montelukast (already in use for asthma), interleukin 13 (IL-13), and statins [327]. Statins, like simvastatin, also seem to act on endothelial nitric oxide synthase (eNOS) to restore nitric oxide production [327]. As for inductors of HbF production, several are being studied in addition to HU (e.g. butyrates, pomalidomide) [325,330] however, they will probably be more efficient when used in a combined manner. Anti-sickling agents include sanguinate and SCD-101 whereas warfarin or prasugrel are among the anticoagulants/antiplatelet drugs [326,328,331,332]. Given the multitude of effects caused by SSRBCs, the more adequate strategy will probably be a multitargeted approach [333]. Unfortunately, a limited number of SCD patients in developed countries and scarce resources in underdeveloped countries make it difficult to accomplish definitive studies of many pharmacological agents [334].

As for emerging genetic-based therapeutic strategies (described earlier), significant improvements are still needed, namely: quality of viral delivery systems; quality and quantity of hematopoietic stem cells harvested; optimization of the gene modification

system in hematopoietic stem cells; choice of recombination pathway (homologous vs non-homologous); identification of the best gene targets; cell manufacturing; preparation regimens to allow the bone marrow to receive genetically modified cells with minimized toxicity; problems related to off-target effects; optimization of preclinical models for testing of developing gene therapy strategies; and parameters that should be used to define cure [321].

Research is also being performed on nutritional-based therapy, with micronutrient supplementation [335] however, the pieces of evidence are still few and further analysis over varying treatment periods and doses of supplements are needed before any conclusions are drawn.

All of these potential therapies are highly dependent on the identification of biomarkers of the different pathways that are predictive of significant clinical response [334].

1.4.5. Genetic Modulation of Cerebral Vasculopathy in SCA

As stated earlier the standard of care in stroke prevention is TCD screening followed by regular blood transfusion therapy for children identified as being at high risk. Unfortunately, the relationship between TCD velocities and stroke incidence is not precise nor age-independent. Approximately seven children with an elevated TCD velocity value have to be treated with regular blood transfusion to prevent one child from having a stroke [186]. Stroke risk also seems to be age-dependent as individuals with ages above sixteen years and elevated TCD velocities do not appear to have a significant increase in stroke risk [336]. Furthermore, the management of hematological disease does not seem to prevent vasculopathy progression, which indicates that additional genetic risk factors have a role as risk modulators. This assumes particular

importance since heterogeneity in disease presentation and severity complicates prognostication, management, and clinical trials [301].

Several biomarkers of cerebral vasculopathy have already been identified such as low HbF level [337], low baseline hemoglobin, high leukocyte count, male sex, and relative high systolic pressure [181,248]. Moreover, high levels of HbF and co-inheritance of α-thal are recognized modifiers of global disease severity [147,338]. Several studies indicated that gene variants co-inherited with the β^S mutation are potential modifiers of stroke/stroke_risk [339–348].

Using approaches that ranged from candidate genes to genome-wide (GWAS) association studies, research on this subject has addressed the issue of identifying loci that might modulate stroke/stroke-risk [339–342,344]. Those genetic modifiers, or modulators, would be potential targets for increasingly accurate (and possibly personalized) prognostic tests and therapeutic strategies. The results together with the previously demonstrated protective role of the concomitant inheritance of α-thalassemia, indicate that the most powerful genetic modifiers of disease severity lie within the globin gene clusters [301].

The candidate gene association study approach requires previous biological and functional knowledge of the genes which is a major drawback [301,349]. However, the strongest disadvantage is also its greatest advantage, as it provides biological context to the variants identified in each gene. Several putative stroke-associated variants have been identified in several genes using this strategy, namely in *VCAM1* [343,344], *IL4R* [344], *LDLR* [344], *ADRB2* [344], *AGT* [346], *HLA* [345], *TNF-*α [344,348], and the -$\alpha^{3.7kb}$-thal deletion [348] (Table 1.1). To circumvent the main limitation of the previous approach genome-wide association studies (GWAS) were devised to offer an unbiased strategy, through scanning thousands to millions of single nucleotide polymorphisms

(SNPs) to find an association with a disease trait [301]. Although promising, GWAS application is hindered by several factors, namely: sample size (which is usually limited); issues with linkage disequilibrium (because GWAS often relies on association of an allele which is inherited concomitantly with another); interpreting associations with SNPs when a biological connection is unclear; and the effects of gene-gene and gene-environment interactions [301]. None of the GWAS has validated any of the associations found in the candidate gene association studies. This probably results from the stringency of usually, 10^{-8}, needed to consider an association by GWAS as significant, which is difficult to obtain in small sample sizes or where the SNP has a large size effect [301].

GWAS are also inadequate to scan for variants with very low frequencies which are better assessed by next-generation sequencing (NGS) strategies. These may be applied to the whole genome (WGS), exome (WES), or transcriptome (RNA-seq). WES has been used to scan for variants associated with pediatric stroke in SCA [342]. That approach led to the identification of 294 SNPs and 6 insertion-deletion variants which included 11 variants within 250 kb of at least 1 SNP identified by GWAS as correlating with stroke risk.

Three SNPs were of special significance: rs662 (Q192R) in the *PON1* gene, rs1044498 (K173Q) in the *ENPP1* gene, and rs3732410 (Y1212C) in the *GOLGB1* gene (Table 1.1). Of these only the SNP on *PON1* had been previously associated with increased stroke risk in adults [350,351]. *ENPP1* – validated also in another study [339] – and *GOLGB1* still lack further confirmation and functional assessment to provide knowledge of their involvement in stroke pathophysiology. These final steps would be vital to consider them potential targets for drug discovery.

Table 1.1. Candidate genes and respective variants, previously identified as putative modifiers of stroke in sickle cell anemia

Gene	Variant	Predicted Modulation	Reference
VCAM1	G1238C	Stroke protection	[343]
VCAM1	T1594C	Increased small-vessel stroke risk	[344]
IL4R	S503P	Increased large-vessel stroke risk	[344]
LDLR	Ncol +/-	Increased small-vessel stroke risk	[344]
ADRB2	Q27E	Increased large-vessel stroke risk	[344]
AGT	AG repeats		[346]
HLA	DRB1*0301	Increased stroke risk	[345]
	DRB1*0302	Increased stroke risk	
	DQB1*0201	Increased stroke risk	
	DRB1*1501	Decreased stroke risk	
	DQB1*0602	Decreased stroke risk	
	DPB1*0401	Increased small-vessel stroke risk	
	DPB1*1701	Increased small-vessel stroke risk	
	-A*0102	Increased large-vessel stroke risk	[352]
	-A*2612	Increased large-vessel stroke risk	
	-A*3301	Decreased large-vessel stroke risk	
TNF-α	-308G>A	Increased stroke risk	[344,348,353]
GOLGB1	Y1212C	Decreased stroke risk	[342]
ENPP1	K173Q	Decreased stroke risk	[342]
		Increased stroke risk	[339]
		Increased stroke risk	[340]
PON1	Q192R	Increased stroke risk	[342]
HBA	-$\alpha^{3.7kb}$ del	Decreased stroke risk	[348]

Like the previous genomic strategies for genetic modulator identification, NGS strategies have limitations. One example is the coverage of WES that only targets 1% of the genome which leaves out most of the SNPs. This is of particular importance since variation in regulatory regions has proven to be determinant for some traits, like

HbF [301]. Our group has also identified a haplotype of *VCAM1* promoter variants with a strong association with hemolysis [354] and stroke [355].

A clearer understanding of the specific mechanisms and genetic variants modulating SCA subphenotypes would be invaluable. This would be particularly important for cerebral vasculopathy, namely in the pediatric setting, due to its impact on patients' life. The possibility of defining a genetic marker panel associated with disease severity would allow for: i) risk stratification of patients – together with the ability to provide timely care to high-risk patients; ii) identification of therapeutic targets – thus facilitating the design of new pharmacological agents; and iii) potentially customize therapeutic strategies to each patient in a precision medicine approach.

It is vital to analyze populations from areas where SCA is more prevalent, and take into account their population-specific genetic modifiers. It is also important to carefully consider ancestry in admixed populations for a correct interpretation of genetic studies. This will additionally provide further possibilities in terms of treatment design and availability.

2. THESIS OBJECTIVES

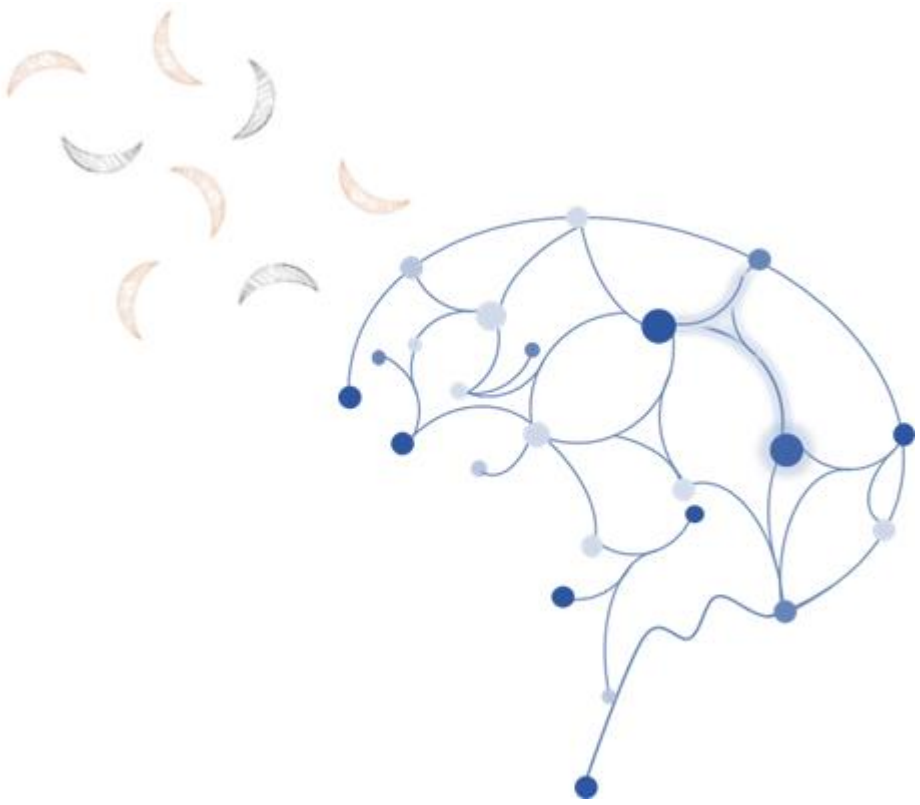
The study described herein aimed to investigate the pathophysiology of cerebral vasculopathy in children with SCA, from molecular mechanisms toward potential therapeutic targets. To do so, the work was subdivided into the following objectives:

- To uncover hematological, biochemical, and genetic biomarkers of pediatric cerebral vasculopathy in SCA– which may be further used for the development of timely and more accurate strategies for preventive therapeutic interventions, before irreversible brain injury (Chapters 2 and 3);

- To validate, through *in vitro* expression studies, namely gene reporter assays, our previous hypothesis of *VCAM1* promoter haplotypes acting as modulators of SCA chronic hemolysis severity, and also of cerebral vasculopathy – that will allow assigning a potential therapeutic target status to *VCAM1* and the pathway(s) where it is involved (Chapter 4);

- To evaluate the differential endothelial expression of genes, identified above, involved in specific pathways. This will potentially allow to predict and monitor the development of cerebral vasculopathy, namely stroke and SCIs in children with SCA (Chapter 4);

- To investigate HU pharmacological rescue of endothelial activation and dysfunction, which will improve the knowledge of the precise action mechanism of this widespread drug on differential endothelial gene expression (Chapter 4).



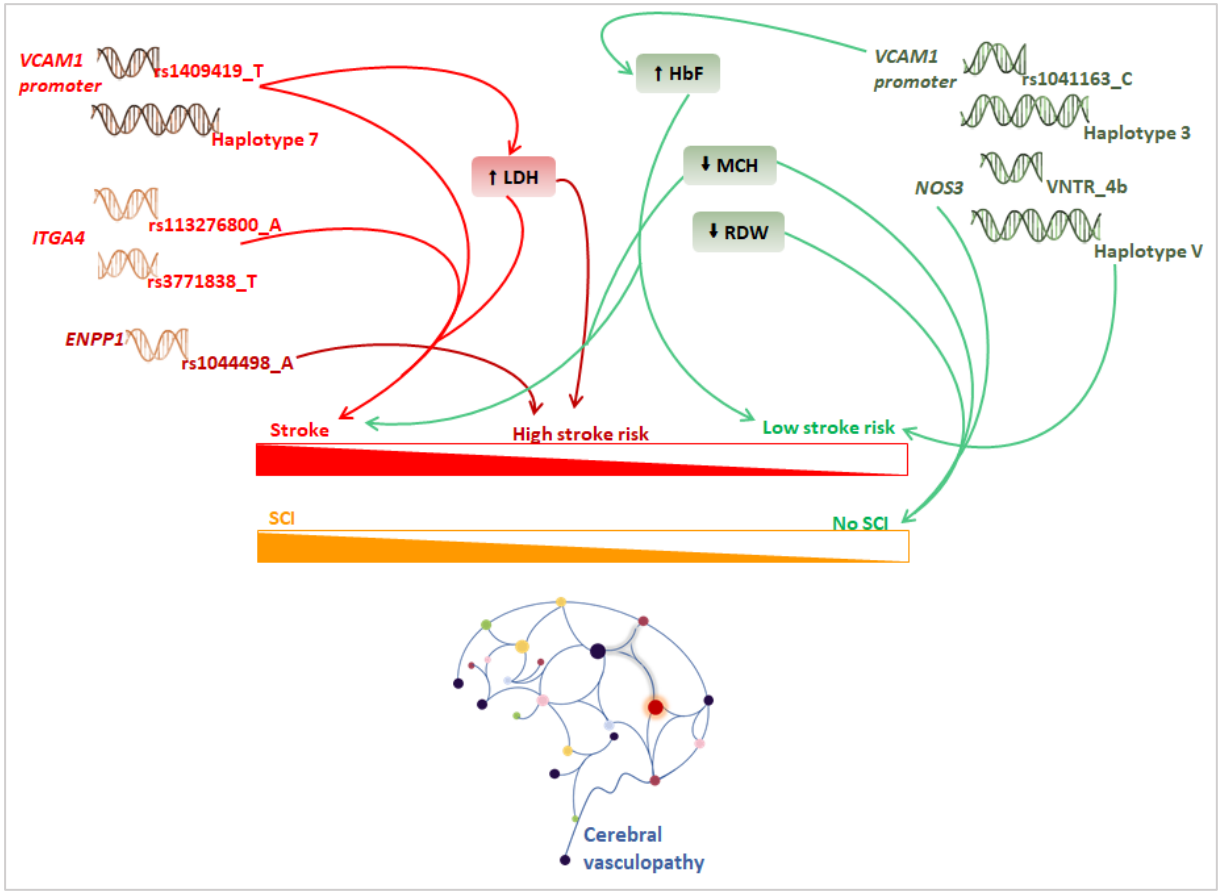
Biomarkers, Genetic Variants and their Predicted Effect in Cerebral Vasculopathy

Part II

Chapter 2

Genetic Modulators and Biomarkers of Cerebral Vasculopathy in Children of Sub-Saharan Ancestry with Sickle Cell Anemia

Graphical abstract



Based on:

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2.1. SUMMARY

This study aimed to investigate biomarkers and genetic modulators of the cerebral vasculopathy sub-phenotype in pediatric SCA patients of Sub-Saharan African ancestry. We found that one *VCAM1* promoter haplotype (haplotype 7) and *VCAM1* single nucleotide variant rs1409419_T were associated with stroke events, stroke risk, as measured by time-averaged mean of maximum velocity in the middle cerebral artery, and with high serum levels of the hemolysis biomarker lactate dehydrogenase. Furthermore, VCAM-1 ligand coding gene *ITGA4* variants rs113276800_A and rs3770138_T showed a positive association with stroke events. An additional positive relationship between a genetic variant and stroke risk was observed for *ENPP1* rs1044498_A. Conversely, *NOS3* variants were negatively associated with silent cerebral infarct events (VNTR 4b_allele and haplotype V) and cerebral vasculopathy globally (haplotype VII). The -$\alpha^{3.7kb}$–thal deletion did not show association with cerebral vasculopathy. However, it was associated with higher red blood cell and neutrophil counts, and lower mean corpuscular volume, mean corpuscular hemoglobin, and red cell distribution width.

Our results underline the importance of genetic modulators of the cerebral vasculopathy sub-phenotype and their potential as SCA therapeutic targets. We also propose that a biomarker panel comprising biochemical, hematological, imaging, and genetic data would be instrumental for cerebral vasculopathy prediction, and prevention.

Keywords: Sickle cell anemia, cerebral vasculopathy, ischemic stroke, genetic modulators, biomarkers.

2.2. BACKGROUND

As stated earlier, SCA is the most common and severe presentation of SCD. High birth rates and child mortality are most frequent in Sub-Saharan Africa, however, population movements are leading to a wider distribution, emphasizing the global health emergence status of the disease [356–358]. In children, the most common sub-phenotypes are cerebral vasculopathy, acute chest syndrome, hyposplenism, renal disease, and painful crises. Cerebral vasculopathy is a major complication and comprises overt stroke, SCIs, transient ischemic attacks, and frequently neurocognitive complications at a later stage. Children with SCA have a much higher risk of stroke than the general pediatric population. As noted earlier, the prevalence of overt stroke approaches 11% by age 20 years. On the other hand, SCIs have been found in up to 37% of children with SCA [181]. The current standard of care for stroke prevention in SCA children is TCD screening – through measurement of the TAMMV in the middle cerebral artery – followed by regular blood transfusions therapy and HU treatment. Despite its high sensitivity, TCD still does not allow identification of all SCA children that will experience a stroke and, conversely, children with high TAMMV (> 200 cm/s) may not develop stroke [283]. Moreover, blood transfusion/HU therapies are not without limitations or adverse side effects [359]. On the other hand, although diagnosis with MRI, or MRA, is recommended for early diagnosis of SCIs and recognition of large vessel stenosis, MRI/MRA are not useful to identify patients at risk of developing SCIs or large vessel stenosis [340]. A more specific and sensitive panel of biomarkers for cerebral vasculopathy prediction and prognosis, that includes genetic variants with disease-modifying effects, is therefore of the utmost importance. In previous studies, we identified variants in *VCAM1*, *NOS3,* and *HBA* with a positive association with chronic

hemolysis, a known pathophysiological SCA mechanism [354]. Building on those results we aimed, in this work, to assess if those variants were also associated with pediatric cerebral vasculopathy in a Sub-Saharan SCA population. Our candidate gene approach also focused on the VCAM-1 α4 integrin ligand gene, *ITGA4*, and for comparison purposes, we included the three genetic variants (*PON1* rs662, *ENPP1* rs1044498, and *GOLGB1* rs3732410) previously reported in association with pediatric stroke in SCA patients [339,340,342].

2.3. METHODOLOGY

2.3.1. Ethical Statement

Ethical approval for the study was granted by the institutional review boards of Instituto Nacional de Saúde Dr. Ricardo Jorge (INSA) and of participant hospitals, in line with the principles of the Declaration of Helsinki. The aim and study procedures were explained to the children's parents (or legal guardians) and they provided informed written consent prior to their enrolment in this study. Due to the sensitive nature of the question asked in this study, survey respondents were assured raw data would remain confidential and would not be shared.

2.3.2. Study Population

This case-control study was performed at INSA in cooperation with four hospitals in the Greater Lisbon area – Hospital D. Estefânia, Hospital de Santa Maria, Hospital Prof. Doutor Fernando Fonseca, and Hospital Garcia de Orta, the four largest centers of that metropolitan area. These centers combined receive the highest numbers of SCA

pediatric patients in our country. Seventy unrelated pediatric patients (≥3 years old) of direct Sub-Saharan African ancestry diagnosed with SCA were selected. Exclusion criteria included age<3 years old, non-African ancestry, previous HU treatment, or having received a blood transfusion in the 120 days before enrolment.

Data obtained from patients and parents (or legal guardians) interviews, which included demographic characteristics (age, gender, parents' geographic origin), were collected. Hemoglobin profiles (HbS, HbF), hematological parameters (RBC, leukocyte, neutrophil and platelet counts, MCV, MCH, RDW) as well as hemolysis biochemical and hematological biomarkers (serum LDH, total bilirubin, and reticulocyte count) were retrospectively collected from patients' hospital records. All these parameters were obtained by standard procedures and HbF levels, in particular, were measured by high-performance liquid chromatography (HPLC). The data collected for each parameter were the result of, at least, three different time-point measurements, performed in steady-state periods, and prior to any treatment with HU and more than 120 days after receiving a blood transfusion.

2.3.3. Genotyping

Genomic DNA was isolated from peripheral blood samples of each patient using the MagNA Pure LC Instrument (Roche Diagnostics GmbH, Mannheim, Germany). All samples were anonymized and specific genotypes could be linked to phenotypes only through the main study database.

The homozygous status for the SCA mutation in the *HBB* gene (c.20A>T) was confirmed by polymerase chain reaction followed by restriction fragment length polymorphism analysis (PCR-RFLP) with the endonuclease *Bsu3*6 I. Beta-globin

cluster haplotypes were determined after examining six restriction endonucleases sites within the cluster: *Xmn* I (5′ to HBG2), *Hin*d III (within the *HBG2* and *HBG1*), *Hin*c II (within and 3′ to *ψHBB*) and *Hin*f I (3′ to *HBB*). Aliquots of the amplified products were digested with the appropriate enzymes under the conditions recommended by the manufacturers. The -$\alpha^{3.7kb}$–thal deletion was assessed by gap-PCR [360].

Putative modifier genes were identified through previous studies by our group [354] and from other published reports based on the influence on the phenotypes of interest. These candidate genes were used to identify and genotype SNPs and other variants in patient samples. For *VCAM1*, *NOS3*, *PON1*, *ENPP1,* and *GOLGB1* genes genotyping was performed using PCR with commercially available or customized primers.

2.3.4. Screening for *ITGA4* Variants by Next-Generation Sequencing

In order to search for variants in the regulatory region of the *ITGA4* gene, next-generation sequencing (NGS) analysis was used on a long PCR fragment (4.1 kb), including its flanking regions. PCR was performed using the primers Fw5'-CAG AGGCTCATTAGGACCC-3′ and Rv5′-CCTTGCGGTACTATCCAGGC-3′ and the FailSafe enzyme with the PreMix G (Epicentre, Illumina, San Diego).

The NGS workflow consisted of five steps: PCR product purification using paramagnetic beads (Agentcourt, Ampure XP); double-stranded DNA quantification in a Qubit fluorometer; DNA library preparation using the Nextera XT kit (Illumina, San Diego) following the manufacturer's instructions; high throughput sequencing in a MiSeq benchtop sequencer (Illumina, San Diego); data analyses were performed using the following tools: Sequencing Analysis Viewer (v1.8.46, Illumina) and FastQC

(v0.11.5, Babraham Bioinformatics, https://www.bioinformatics.babraham.ac.uk/projects/fastqc) were used for quality analysis. The MiSeq® Reporter software package (v2.6.2, Illumina) was used for read mapping (with Burrows-Wheeler Aligner) and variant calling and filtering (with Genome Analysis Toolkit). FastQ screen (v.0.9.3, Babraham Bioinformatics) was used to screen for contamination between samples. Variant Effect Predictor (www.ensembl.org) was used to annotate variants and Integrative Genomics Viewer (v.2.3.86) [Broad Institute; [361]] was used for visualization of reads and variants. Validation of the variants was performed using automated Sanger sequencing, after amplification with customized primers in the 3130XI Genetic Analyser, (Applied Biosystems). The results were analyzed using FinchTV v.1.4.0 software (Geospiza, Inc.). The genotyping results were added to the previously created database.

2.3.5. Haplotype Reconstruction

Beta-globin cluster haplotypes reconstruction was based on the presence or absence of cleavage at the *Xmn* I, *Hin*d III, *Hin*c II, and *Hin*f I sites. Haplotypes were determined by comparison of the compiled pattern with known Bantu, Benin, and Senegal patterns [362].

Haplotype reconstruction for the other genes was performed using PHASE software v2.1 developed by Mathew Stephens at Washington University, according to the developer's instructions (https://els.comotion.uw.edu/express_license_technologies/phase). Haplotypes were reconstructed for genetic variants in *NOS3* (rs2070744, intron 4_27 bp VNTR, rs1799983), in the promoter of *VCAM1* (rs1409419, rs3917024, rs3917025, rs3783598, rs1041163, rs3783599) and for genetic variants of *ITGA4* (rs1375493, rs35723031, rs10562650; rs1839269 and rs1839268).

2.3.6. *In Silico* Analysis

Population allele and genotype frequencies were recorded for each observed variant using the Ensembl browser (www.ensembl.org). SNPs' sequences were retrieved using the NCBI SNP search engine (http://ncbi.nlm.gov/snp).

Transcription factor binding site analysis was performed for the variants identified in the regulatory regions, using the MatInspector tool (Genomatix, Munich, Germany), to evaluate potential effects on the regulation of gene expression by altering putative transcription factor binding sites. Only results above the 0.85 threshold were considered, which corresponds to a maximum of 15% dissimilarity between the identified sequence and the consensus sequence. A comparison with previously reported consensus sequences of transcription factor binding sites for the *VCAM1* [124] and the *ITGA4* [363] promoters was performed. The sequences of the identified variants were not found to overlap with any of the previously reported transcription factor binding sites' consensus sequences. Hence, no marked effects for these genes' expression are to be expected as a result of the presence of those variants.

Detailed examples of the *in silico* analyses are provided in chapter 3.

2.3.7. Statistical Analysis

The analyses were performed using the SPSS software (version 25.0, IBM Inc., Chicago, USA). For descriptive analysis, continuous variables were represented as medians and interquartile ranges (IQR). To evaluate the Gaussian distribution of variables, Shapiro-Wilk normality tests were applied. We used the Mann-Whitney U test to compare the medians of variables. Categorical variables are represented as numbers, frequencies, and percentages. The chi-square test or the Fisher's exact test

were used to compare categorical variables on bivariate analysis. Statistical significance was defined as $p < 0.05$.

The minor allele for each variant was evaluated for potential association with stroke, SCI, cerebral vasculopathy (stroke and SCI combined), or risk (as measured by TCD-TAMMV values), via 2 × 2 phenotype × genotype contingency tables. Only polymorphisms with a minor allele frequency (MAF) > 5% were considered for association analysis. Phenotypes were classified as follows: (i) “stroke” (at least one previous overt ischemic stroke event, as confirmed by MRI/MRA) or “no stroke” (no clinically/imaging identified stroke event); (ii) “SCI” (at least one event as identified by MRI/MRA) or “no SCI” (no SCI events, as confirmed by MRI); (iii) “cerebral vasculopathy” (at least one overt ischemic stroke and/or SCI event) or “no cerebral vasculopathy”; and (iv) “risk of stroke” (high/moderate: TAMMV ≥ 200 cm/s or 199 ≥ TAMMV ≥ 170 cm/s; low: TAMMV < 170 cm/s).

Each variant was also evaluated for potential association with biochemical and hematological parameters, including hemolysis biomarkers (LDH, total bilirubin, reticulocyte count).

2.4. RESULTS

2.4.1. Population Description and Genotyping

This study was performed on seventy unrelated SCA patients, (age range: 3–16 years, 40 males, 30 females), living in Portugal but of direct Sub-Saharan ancestry, with parental geographic origin mainly from Angola, São Tomé and Príncipe, and Cape Verde (Table 2.1).

A total of seventy-one genetic variants were identified of which twenty-eight (MAF > 0.05) were used in the association studies – seven in *VCAM1*, five in *NOS3*, three in *GOLGB1*, one in *PON1*, one in *ENPP1*, one in *HBA2* (-$\alpha^{3.7kb}$–thal) and ten in *ITGA4* (Supplementary Table S2.1). We were able to reconstruct sixteen haplotypes and used ten of them (frequency > 0.05) for statistical analysis. Concerning the *HBB* gene cluster haplotypes, only the more frequent genotypes, Bantu/Bantu and Senegal/Senegal, were used for statistical analysis (frequency > 0.05).

Table 2.1. Demographic, neurological status, and laboratory parameters of the population in this study

Age (years)		3 – 16	
Gender		***n***	**%**
Male		40	57.1
Female		30	42.9
Parental Geographic Origin		***n***	**%**
Angola		42	60.0
São Tomé and Príncipe Islands		8	11.4
Cape Verde		5	7.1
Guinea-Bissau		7	10.0
Guinea-Conakry		1	1.4
Nigeria		1	1.4
Double origin		6	8.6
Neurological Status (*n* = 70)		***n***	**%**
Stroke (*n* = 62)	Yes	15	24.2
	No	47	75.8
SCI (*n* = 53)	Yes	9	16.9
	No	44	83.0
Cerebral vasculopathy (*n* = 62)	Yes	24	38.7
	No	38	61.3
Stroke risk (*n* = 60)	High (TAMMV≥200 cm/s)	25	41.7
	Moderate (170 cm/s ≤ TAMMV ≤ 200	6	10.0
	Low risk (TAMMV<170 cm/s)	29	48.3
Hematological Parameters		**Median**	**IQR**
Hb S (%)		79.9	14.5
Hb F (%)		10.7	11.7

Hb (g/dL)	8.0	1.3
RBC ($x10^{12}$/L)	3.0	0.7
MCV (fL)	81.3	14.3
MCH (pg)	26.9	6.0
Reticulocytes (%)	9.9	6.3
RDW (%)	21.2	4.5
Platelets ($x10^9$/L)	404.1	167.7
WBC ($x10^9$/L)	12.6	4.8
Neutrophils ($x10^9$/L)	5.6	2.65
Biochemical Parameters	**Median**	**IQR**
LDH (U/L)	636.3	473.4
Total bilirubin (mg/dL)	2.6	1.9
***HBB* cluster haplotype**	***n***	**%**
Bantu/Bantu	38	54.3
Senegal/Senegal	11	15.7
Benin/Benin	3	4.3
Compound heterozygous	17	24.2
Atypical	1	1.4

2.4.2. *In Silico* Analysis

In silico analysis of the *VCAM1* gene promoter variants was focused on those with MAF > 0.05, except rs3917025 due to its occurrence in only one haplotype (Haplotype 3). The rs1041163_C, rs1409419_T, and rs3917025_delCT *VCAM1* alleles were classified (according to ClinVar and Ensembl's VEP and Mat Inspector) as intergenic variants with potential modifying impact, although with no major pathologic effects. Potential changes resulting from the presence of the rs1041163_C allele include (i) alteration of the transcription factor binding site for a retinoid X receptor heterodimer binding site (RXRF), substituting it for a PR domain zinc finger protein 1 (PRDM1) site, and (ii) loss of a forkhead homologous X (FHXB) binding site. The presence of rs1409419_T would lead to a potential gain of binding sites, in particular, for ecotropic virus integration site-1 (EVI-1), octamer-binding transcription factor (Oct1), and homeobox protein BarH-like 2 (BarX2).

Regarding rs3917025_delCT, a potential gain of a forkhead activin signal transducer-1 (FAST1) binding site was indicated.

2.4.3. Association of Biochemical and Hematological Parameters with Cerebral Vasculopathy

We observed significant associations of both stroke and stroke risk with several biochemical and hematological parameters (Table 2.2). Lower HbF percentages and MCH values were positively associated with stroke, while stroke risk was associated not only with lower HbF percentage but also with higher levels of coagulation, inflammation, and hemolysis markers.

Table 2.2. Association of biochemical and hematological parameters of SCA patients with stroke and stroke risk

Parameter (units)		**Stroke**		
		Yes	**No**	
	n	Medians (IQR)	Medians (IQR)	***p***
HbF (%)	64	3.2 (9.3)	11.9 (10.3)	0.018
MCH (pg)	70	21.2 (20.8)	27.4 (6.0)	0.005
		Stroke Risk		
		High + Moderate	**Low**	
		Medians (IQR)	Medians (IQR)	
HbF (%)	64	8.5 (10.2)	12.1 (10.7)	0.043
Platelets (x10^9/L)	61	442.0 (156.4)	363.2 (124.2)	0.017
Neutrophils (x10^9/L)	61	6.3 (2.5)	4.9 (2.6)	0.009
LDH (U/L)	65	761.5 (535.7)	510.0 (325.3)	<0.001

2.4.4. Association of Genetic Variants with Biochemical and Hematological Parameters

Genetic variants analyzed in our study, using the dominant genetic test model, showed association with both hematological and biochemical parameters, whether individually or as part of specific haplotypes (Table 2.3). *VCAM1* rs1041163 (CC + TC), *VCAM1*_haplotype 3, *ITGA4* rs113276800 (CA) and *ITGA4* rs3770138 (TT + CT) showed an association with lower levels of HbS. Conversely, higher HbF percentages were observed in association with rs1041163 (CC + TC), *VCAM1*_haplotype 3, and with Senegal/Senegal haplotype.

Namely, *VCAM1* as well as *ITGA4* variants, individually or within a haplotype context, were significantly associated with traditional biomarkers of disease severity, such as lower HbS percentage and higher LDH and total bilirubin values.

As for *PON1* rs662, the AA and GA genotypes showed a positive association with high platelet counts characteristic of a pro-coagulant state. Regarding *GOLGB1*, no significant association was found between the presence of rs3742410_C and hematological or biochemical parameters. However, we found two other *GOLGB1* SNPs while analyzing rs3732410_C – rs61746571_G and rs33988592_A, a synonymous and a missense variant, respectively. The rs61746571_G seems to be in linkage disequilibrium with variant rs3732410_C. On the other hand, rs33988592 AA and GA genotypes showed an association with lower values of inflammation markers (Table 2.3).

Table 2.3. Genetic variants' association with the hematological and biochemical parameters

Gene	Variant ID	Allele change or haplotype	Parameter (unit; nr. patients)		*p*
			HbS (%; n=60)		
			Var	***No var***	
VCAM1	rs1041163	T>C	74.5	83.5	0.019
	Haplotype 3	C/C/CT/T/C/C	73.7	81.4	0.034
	rs33988592	G>A	11.3	13.4	0.030
ITGA4	rs113276800	C>A	69.0	80.4	0.012
	rs3770138	C>T	66.1	80.7	0.003
			HbF (%; n=64)		
			Var	***No var***	
VCAM1	rs1041163	T>C	13.0	7.0	0.014
	Haplotype 3	C/C/CT/T/C/C	14.5	9.3	0.005
HBB	Haplotype	Sen/Sen	13.6	9.2	0.038
			Hb (g/dL; n=65)		
			Var	***No var***	
HBB	Haplotype	Sen/Sen	8.1	7.9	0.022
			RBC (x10^{12}/L; n=64)		
HBA	$-\alpha^{3.7kb}$	$\alpha\alpha > -\alpha^{3.7kb}$	3.1	2.7	0.008
			MCV (fL; n=69)		
NOS3	rs2070744	T>C	87.3	79.9	0.024
	Haplotype IV	T/4a/G	78.7	83.2	0.032
HBA	$-\alpha^{3.7kb}$	$\alpha\alpha>-\alpha^{3.7kb}$	75.7	85.7	<0.001
HBB	Haplotype	Sen/Sen	90.1	80.6	0.039
			MCH (pg; n=70)		
			Var	***No var***	
HBA	$-\alpha^{3.7kb}$	$\alpha\alpha>-\alpha^{3.7kb}$	25.1	29.0	0.001
			RDW (n=70)		
			Var	***No var***	
NOS3	rs2070744	T>C	19.6	21.6	0.031
	Haplotype I	C/4a/G	19.6	21.5	0.044
HBA	$-\alpha^{3.7kb}$	$\alpha\alpha>-\alpha^{3.7kb}$	20.8	21.2	0.021

			Platelets (x10^9/L; n=61)		
			Var	***No var***	
PON1	rs662	G>A	442.0	378.1	0.028
			WBC (x10^9/L; n=67)		
GOLGB1	rs33988592	G>A	11.3	13.4	0.030
HBB	Haplotype	Sen/Sen	9.9	13.0	0.030
			Neutrophils (x10^9/L; n=61)		
			Var	***No var***	
GOLGB1	rs33988592	G>A	5.0	6.2	0.013
HBA	-$\alpha^{3.7kb}$	$\alpha\alpha$>-$\alpha^{3.7kb}$	5.7	5.5	0.010
HBB	Haplotype	Bantu/Bantu	6.2	5.0	0.036
	Haplotype	Sen/Sen	4.7	6.2	0.014
			LDH (U/L; n=65)		
			Var	***No var***	
VCAM1	rs1409419	C>T	748.0	517.0	<0.001
	Haplotype 7	T/C/CT/T/T/C	748.0	517.0	<0.001
ITGA4	Haplotype A	G/GA/TT	611.5	1269.0	0.003
			Bilirubin (mg/dL; n=69)		
			Var	***No var***	
VCAM1	rs3783613	G>C	3.2	2.4	0.026

Var – presence of variant allele or haplotype; ***No var*** – absence of variant allele or haplotype; HbS – hemoglobin S; HbF – fetal hemoglobin; Hb – total hemoglobin; RBC – red blood cells; MCV – mean corpuscular volume; MCH – mean corpuscular hemoglobin; RDW – red cell distribution width; WBC – white blood cells; LDH – lactate dehydrogenase; Sen - Senegal

2.4.5. Association of Genetic Variants with Cerebral Vasculopathy and Cerebral Vasculopathy Risk

We found a significant association between the presence of several of the variants identified and CV/CV risk (Table 2.4). Namely, *VCAM1* rs1409419 (TT+CT), haplotype 7, *ITGA4* rs113276800 (CA), and rs3770138 (TT+CT), showed a positive association with stroke. *ITGA4* variant rs3770138 (TT+CT) was also positively associated with CV as a whole.

Table 2.4. Genetic variants association with cerebral vasculopathy

Gene	Variant	**Stroke** (*n* = 62)				
		Yes	**No**	***p***	**OR**	**95% CI**
VCAM1	rs1409419_TT+CT	12	23	**0.041**	4.17	1.04 – 16.73
	rs1409419_CC	3	24			
	Haplotype 7	12	23	**0.041**	4.17	1.04 – 16.73
	Haplotype X	3	24			
ITGA4	rs113276800_CA	4	3	**0.025**	7.62	1.39 – 41.65
	rs113276800_CC	7	40			
	rs3770138_TT+CT	4	4	**0.045**	5.57	1.12 – 27.67
	rs3770138_CC	7	39			
		SCI (n = 53)				
		Yes	**No**	***p***	**OR**	**95% CI**
NOS3	VNTR_4b	6	42	**0.030**	0.10	0.01 – 0.69
	VNTR_4a+4c	3	2			
	Haplotype V	4	36	**0.031**	0.18	0.04 – 0.81
	Haplotype Y	5	8			
		CV (n =62)				
		Yes	**No**	***p***	**OR**	**95% CI**
NOS3	Haplotype VII	1	23	**0.006**	0.08	0.01 – 0.69
	Haplotype Z	13	25			
		Stroke risk (n=60)				
		High+Moderate	**Low**	***p***	**OR**	**95% CI**
VCAM1	rs1409419_TT+CT	23	11	**0.009**	4.71	1.57 – 14.13
	rs1409419_CC	8	18			
	Haplotype 7	23	11	**0.009**	4.71	1.57 – 14.13
	Haplotype X	8	18			
NOS3	Haplotype V	18	24	**0.050**	0.29	0.09 – 0.96
	Haplotype Y	13	5			
ENPP1	rs1044498_AA+CA	14	5	**0.026**	4.03	1.21 – 13.42
	rs1044498_CC	16	23			

Haplotype X – presence of any of the other *VCAM1* promoter haplotypes studied; Haplotype Y – presence of any of the other *NOS3* haplotypes studied; SCI – silent cerebral infarction; CV – cerebral vasculopathy; 95% CI – 95% confidence interval.

Positive associations were also found between high TAMMV values and *VCAM1* rs1409419 (TT+CT), *VCAM1* promoter haplotype 7, and also with the *ENPP1* rs1044498_A allele. On the other hand, *NOS3* intron 4 VNTR_4b allele and haplotype V were negatively associated with SCI, while haplotype VII showed a negative association with CV overall.

2.5. DISCUSSION

Our study aimed to assess demographic, clinical, biochemical, hematological, genotyping and imaging data to design a potential biomarker panel for cerebral vasculopathy prognosis in children with SCA. With this approach, we were able to identify statistically significant associations of biochemical, as well as hematological parameters, with genetic variants and cerebral vasculopathy.

In silico analysis of the *VCAM1* rs1409419_T allele indicated particularly interesting potential transcription factor binding site gain, namely for EVI-1, Oct1, and BarX2 transcription factors. EVI1 is of special note due to its complexity, multiple targets, and modulation of several numerous processes, including cell migration, adhesion, and proliferation. It may cooperate with the FOS transcription factor to limit cell adhesion while enhancing cell proliferation [364]. Conversely, Oct1 is a transcription factor known to promote a transcriptional repression/silencing effect, which would potentially result in *VCAM1* down-regulation. On the other hand, a BarX2 binding site gain, predicted as a result of the rs1409419_T presence, has been shown to promote murine muscle cell differentiation, by interacting with muscle regulatory factors, whereas its loss would lead to decreased adhesion properties [365]. Hence, it is reasonable to assume that a gain could result in increased adhesion properties. All the transcription factors whose

expression may be affected by the significantly associated variants are mainly involved in development and in different tissues following the proposed role of VCAM-1 [110]. It is important to emphasize that while we can address the potential effects of the individual *VCAM1* variants' genotypes, the most important modifying role on disease manifestation would probably arise in the context of the haplotypes that encompass them. The one exception seems to be rs1409419_T given the overlapping findings observed for this variant and haplotype 7, which includes it.

We found that variants in the gene that encodes the α4 integrin (VCAM-1 ligand), *ITGA4*, namely rs113276800_A and rs3770138_T were also positively associated with stroke. Furthermore, the latter was associated with cerebral vasculopathy globally. Given its role in WBC, reticulocyte, and sickle erythrocyte adhesion to the activated SCA endothelium, this finding further underlines the role of cell-endothelium adhesion in the severe cerebral vasculopathy sub-phenotype. *ITGA4* rs113276800 has been previously described in association with multiple sclerosis and, as in our case, no AA individuals were observed [366,367]. This variant is located in the *ITGA4* promoter region near the activator protein-2 (AP-2) transcription factor binding sites and the AA genotype may, therefore, cause a negative expression of the integrin α4 subunit [368]. We also found that the *ITGA4* rs1375493, rs35723031, and rs10562650 variants behaved similarly in our group of patients, most of whom were heterozygous for the three of them simultaneously. Furthermore, the co-occurrence of minor alleles in haplotype A – which, to our knowledge, has not been previously described – was associated with lower LDH values and potentially to a protective effect against hemolysis. eNOS is the major NO-producer enzyme in the cardiovascular system, playing a crucial role in vascular tone control, vascular remodeling, and proliferation. Furthermore, in SCA, NO bioavailability plays a very important modulating role,

primarily through scavenging by cell-free hemoglobin [171]. The rs2070744 variant, located at position −786 of the *NOS3* gene 5′ flanking region, has been correlated with cardiovascular disease, namely its C allele, although there is still debate about how it affects mRNA and protein levels. In our study, we did not find any association of this variant with cerebral vasculopathy or stroke risk, which may be in accordance with previous reports of no significant differences between CC and TT genotypes effect on *NOS3* promoter activity [369]. The fact that the rs2070744_C shows different distributions in different ethnic backgrounds [370] may also be responsible, to some extent, for these differences, since that allele is more frequent in Caucasians and our study population is of Sub-Saharan origin. However, we observed a significant association between CC and TC genotypes of this variant and lower RDW, which has been discussed as a possible biomarker of lower cerebrovascular disease risk [371]. Lower RDW values (or reduced anisocytosis) would potentially act as a protective factor in consonance with what we observed for *NOS3* haplotype V, which includes allele C, and cerebral vasculopathy protection. Although rs2070744 has been described in association with cardiovascular disease [372], its role in ischemic stroke has not been consensual. *NOS3*_haplotype V also includes intron 4 VNTR_4b allele, a variant that showed a protective effect against SCI. However, no relationship with any of the cerebral vasculopathy presentations studied here was observed. On the other hand, the *NOS3* rs1799983_T allele, which leads to aspartate for glutamate substitution at eNOS position 298, has been previously reported to be related to deficient eNOS caveolar localization and deficient shear stress response leading to reduced enzymatic activity. This SNP has been found in some populations to be more prevalent in patients with coronary artery disease, ischemic stroke, and arterial hypertension [373]. However, in our

study population, we did not observe any relationship of rs1799983 TT or GT genotypes with cerebral vasculopathy, biochemical or hematological parameters.

Several studies have identified other candidate gene polymorphisms as potentially affecting the risk of cerebral vasculopathy. However, the results published so far have been conflicting. A GWAS study by Flanagan et al. [342], performed in a large cohort of mainly African American SCA pediatric patients, showed a decreased risk of clinically overt stroke in association with *GOLGB1* rs3732410_G (Y1212C) and *ENPP1* rs1044498_C (K173Q) mutations, whereas *PON1* rs662_C (Q192R) was associated with increased risk of stroke. In the same study, *GOLGB1* Y1212C was associated with reduced TCD velocities and lower frequencies of SCIs. Conversely, reports from Martella et al. [340] and Belisário et al. [339] indicated a link between *ENPP1* rs1044498_A and increased stroke risk as well as high TCD velocities. In our study, the *ENPP1* rs1044498_A allele was found in 18% of patients compared to 68.33% of Martella et al. [340], 26.08% of Belisário et al. [339], and 5.08% of Flanagan et al. [342], while homozygosity for the *GOLGB1* rs3732410_G allele was not found in our patients as in Flanagan's studies but contrary to 1.67% in Martella's report. Homozygotes for the *PON1* rs662_C allele occurred in a frequency of 10,3% in our study, whereas Martella et al. [340] and Flanagan et al. [342] reported 45% and 13.7%, respectively. Of the three variants, only *ENPP1* rs1044498 AA and AC genotypes showed a positive association with stroke risk. Notably, rs1044498_A is the minor allele, in our study group, while the variant allele (C) is the most frequent, which is in line with the reference population (African Yoruba) and contrary to what is described for the other reference populations. This may reflect a negative selection for the less advantageous allele – rs1044498_A in these populations. As for the *PON1* rs662_C variant, albeit no association with stroke or global cerebral vasculopathy was apparent, we observed a positive

association with high platelet levels, indicating a potential impact on hemostasis and inflammation.

The only consensual modifiers of SCD disease severity, so far, have been the persistence of HbF beyond infancy and the presence of -$\alpha^{3.7kb}$–thal deletion. The co-inheritance of -$\alpha^{3.7kb}$–thal and homozygous HbS mutation has been associated with an overall ameliorating effect on anemia, particularly a protective effect against stroke in children [151]. We did not find any direct relationship between the presence of the -$\alpha^{3.7kb}$–thal deletion and stroke, although we did find that patients with α–thal showed a higher RBC count, lower MCV, and MCH, which is consistent with previous reports [374,375]. Other authors have also reported an absence of association between -$\alpha^{3.7kb}$–thal presence and stroke protection [376,377]. Despite the small sample size in our study, we cannot exclude that population heterogeneity or other specific population characteristics may contribute to the lack of association observed. Additionally, the unexpected association with increased neutrophil count might lower the above-mentioned potentially favorable effect by indicating a proinflammatory role. The latter was also found in subjects with the Bantu haplotype while the Senegal haplotype seems to have the opposite effect, ameliorating inflammation and the hematological indices. Nevertheless, in our study, no *HBB* cluster haplotypes were found to be associated with cerebral vasculopathy.

2.6. CONCLUSION

Although the sample size in our study limits extrapolation to the general SCA pediatric population, our results seem to reinforce the importance of genetic modulators in the pathophysiology of cerebral vasculopathy and provide clues for the discovery of novel

targets for SCA therapy. Our findings lead us to suggest that a comprehensive biomarker panel that includes biochemical, hematological, imaging as well as genetic data may be very important for cerebral vasculopathy prediction, and prevention. Even though the physical and social environmental factors affecting the children we studied are different from the ones affecting the populations their parents originated from, the genetic modifiers described in our study may provide further tools for cerebral vasculopathy prevention in SCA. Functional studies are of the utmost importance, not only for confirmation purposes, but also to assess the mechanisms by which the phenotype modulation may occur, and the potential use of these variants as novel genetic biomarkers of disease prognosis.

Acknowledgments

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2.7. SUPPLEMENTARY MATERIAL

Supplementary Table S2.1. Genetic variants included in this study

Gene	Chr	Variant	Alleles	Functional consequence*	Protein change	n**	Minor allele	MAF	MAF reference ***	Genotypes	Genotype / Haplotype Frequency	Genotype Frequency Reference***
VCAM1	1	rs1409419	C>T	Intergenic variant	___	70	T	0.229	0.352 (T)	CC	0.414	0.407
										CT	0.500	0.481
										TT	0.086	0.111
		rs3917024	C>T	Intergenic variant	___	70	T	0.029	0.093 (T)	CC	0.943	0.815
										CT	0.057	0.185
		rs3917025	CT>--	Intergenic variant	___	70	--	0.071	0.120 (--)	CT	0.857	0.759
										CT/--	0.143	0.251
		rs3783598	T>G	Intergenic variant	___	70	G	0.029	0.093 (G)	TT	0.943	0.815
										TG	0.157	0.185
		rs1041163	T>C	Intergenic variant	___	70	C	0.179	0.194 (C)	TT	0.643	0.630
										TC	0.243	0.352
										CC	0.114	0.019
		rs3783599	C>T	Intergenic variant	___	70	T	0.093	0.093 (T)	CC	0.814	0.815
										CT	0.157	0.185
										TT	0.029	___

VCAM1	1	Haplotype 1	rs1409419_C rs3917024_T rs3917025_CT rs3783598_T rs1041163_T rs3783599_C	___	___	70	___	___	___	___	0.282	___
		Haplotype 2	rs1409419_C rs3917024_C rs3917025_CT rs3783598_T rs1041163_T rs3783599_T	___	___	70	___	___	___	___	0.007	___
		Haplotype 3	rs1409419_C rs3917024_C rs3917025_CT rs3783598_T rs1041163_C rs3783599_C	___	___	70	___	___	___	___	0.143	___
		Haplotype 4	rs1409419_C rs3917024_C rs3917025_CT rs3783598_T	___	___	70	___	___	___	___	0.107	___

			rs1041163_C rs3783599_T									
		Haplotype 5	rs1409419_C rs3917024_C rs3917025_-- rs3783598_T rs1041163_T rs3783599_C	___	___	70	___	___	___	___	0.021	___
		Haplotype 6	rs1409419_C rs3917024_T rs3917025_-- rs3783598_G rs1041163_T rs3783599_C	___	___	70	___	___	___	___	0.029	___
VCAM1	1	Haplotype 7	rs1409419_T rs3917024_C rs3917025_CT rs3783598_T rs1041163_T rs3783599_C	___	___	70	___	___	___	___	0.350	___
		rs3783613	G>C	Missense variant	p.Gly413Ala	70	C	0.086	0.167 (C)	GG GC	0.686 0.286	0.685 0.296

									CC	0.029	0.019
		rs10203703	C>T	Upstream transcript variant	___	61	T	0.066	0.019 (T)	CC CT	0.869 0.114	0.954 0.046
		rs12988934	C>T	Non-coding transcript exon variation	___	61	T	0.066	<0.001 (T)	CC CT TT	0.869 0.131 ___	1.000 ___ ___
		rs113276800	C>A	5 prime UTR variant	___	61	A	0.057	0.023 (C)	CC CA	0.885 0.115	0.954 0.046
		rs1375493	G>A	Non-coding transcript exon variant	___	61	G	0.484	0.333 (G)	AA AG GG	0.148 0.738 0.115	0.444 0.444 0.111
ITGA4	2	rs35723031	GA>GTA	Non-coding transcript exon variant	___	61	GA	0.484	0.333 (GA)	GTA/GTA GTA/GA GA/GA	0.148 0.738 0.115	0.444 0.444 0.111
		rs10562650	TT>--	Intron variant	___	61	TT	0.484	0.333 (TT)	TT TT/-- --/--	0.148 0.738 0.115	0.111 0.444 0.444
		Haplotype A	rs1375493_G rs35723031_GA rs10562650_TT	___	___	61	___	___	___	___	0.484	___
		rs1839269	G>T	Intron variant		61	T	0.082	0.046 (T)	GG GT	0.743 0.114	0.907 0.093

										TT	0.016	___
		rs1839268	C>T	Intron variant		61	T	0.082	0.046 (T)	CC CT TT	0.743 0.114 0.016	0.907 0.093 ___
		Haplotype B	rs1839269_T rs1839268_T	___		61	___	0.082	___	___	0.082	___
ITGA4	2	rs3770138	C>T	Intron variant	___	61	T	0.066	0.028 (T)	CC CT	0.869 0.131	0.944 0.056
		rs1143676	G>A	Missense variant	p.Arg878Gln	70	A	0.264	0.204 (G)	GG GA AA	0.514 0.443 0.043	0.028 0.352 0.620
		rs2070744	C>T	5 prime UTR variant	___	70	T	0.143	0.125 (C)	CC CT TT	0.743 0.229 0.029	___ 0.250 0.750
NOS3	7	Intron 4 VNTR_4a	4 repeats x 27 bp	Intron variant	___	70	___	0.329	___	4a/4a 4a/4b 4a/4c 4b/4b 4b/4c 4c/4c	0.043 0.500 0.071 0.243 0.143 ___	___
		Intron 4 VNTR_4b	5 repeats x 27 bp	Intron variant	___	70	___	0.564	___			___

		Intron 4 VNTR_4c	6 repeats x 27 bp	Intron variant	___	70	___	0.107	___			___
		rs1799983	T>G	Missense variant	p.Asp298Glu	70	T	0.093	0.056 (T)	TT TG GG	0.843 0.129 0.029	___ 0.111 0.750
		Haplotype I	rs2070744_C VNTR_4a rs1799983_G	___	___	70	___	___	___	___	0.079	___
		Haplotype II	rs2070744_C VNTR_4b rs1799983_G_	___	___	70	___	___	___	___	0.036	___
NOS3	7	Haplotype III	rs2070744_C VNTR_4b rs1799983_T	___	___	70	___	___	___	___	0.029	___
		Haplotype IV	rs2070744_T VNTR_4a rs1799983_G	___	___	70	___	___	___	___	0.250	___
		Haplotype V	rs2070744_T VNTR_4b rs1799983_G	___	___	70	___	___	___	___	0.307	___
		Haplotype VI	rs2070744_T VNTR_4b	___	___	70	___	___	___	___	0.057	___

			rs1799983_T								
NOS3	7	Haplotype VII	rs2070744_T VNTR_4c rs1799983_G	___	___	70	___	___	___	___	0.107	___
PON1	7	rs662	T>C (A>G)	Missense variant	p.Gln192Arg	68	C (G)	0.301	0.218 (T)	TT TC CC	0.500 0.397 0.103	0.037 0.361 0.602
ENPP1	6	rs1044498	A>C	Missense variant	p.Lys66Gln	68	C	0.176	0.079 (A)	AA CA CC	0.059 0.236 0.706	___ 0.157 0.843
GOLGB1	3	rs3732410	T>C	Missense variant	p.Tyr1212Cys	67	C	0.052	0.051 (C)	TT TC	0.896 0.144	0.898 0.102
		rs61746571	A>G	Synonymous variant	p.Tyr1217=	67	G	0.023	0.088 (G)	AA AG	0.955 0.045	0.824 0.176
		rs33988592	G>A	Missense variant	p.Pro1254Ser	67	A	0.157	0.144 (A)	GG GA AA	0.700 0.224 0.045	0.722 0.269 0.090
HBB	11	Haplotype	Bantu/Bantu	___	___	70	___	___	___	___	0.540	___
		Haplotype	Senegal/Senegal	___	___	70	___	___	___	___	0.157	___
		Haplotype	Benin/Benin	___	___	70	___	___	___	___	0.043	___
		Haplotype	Bantu/Senegal	___	___	70	___	___	___	___	0.043	___
		Haplotype	Bantu/Benin	___	___	70	___	___	___	___	0.114	___

HBB	11	Haplotype	Senegal/Benin	___	___	70	___	___	___	___	0.014	___
		Haplotype	Atypical	___	___	70	___	___	___	___	0.086	___
HBA	16	Haplotype	$\alpha\alpha$>-$\alpha^{3.7kb}$		___	70	-$\alpha^{3.7kb}$		___	-$\alpha^{3.7kb}$/-$\alpha^{3.7kb}$	0.057	
										$\alpha\alpha$/-$\alpha^{3.7kb}$	0.343	___
										$\alpha\alpha$/$\alpha\alpha$	0.600	

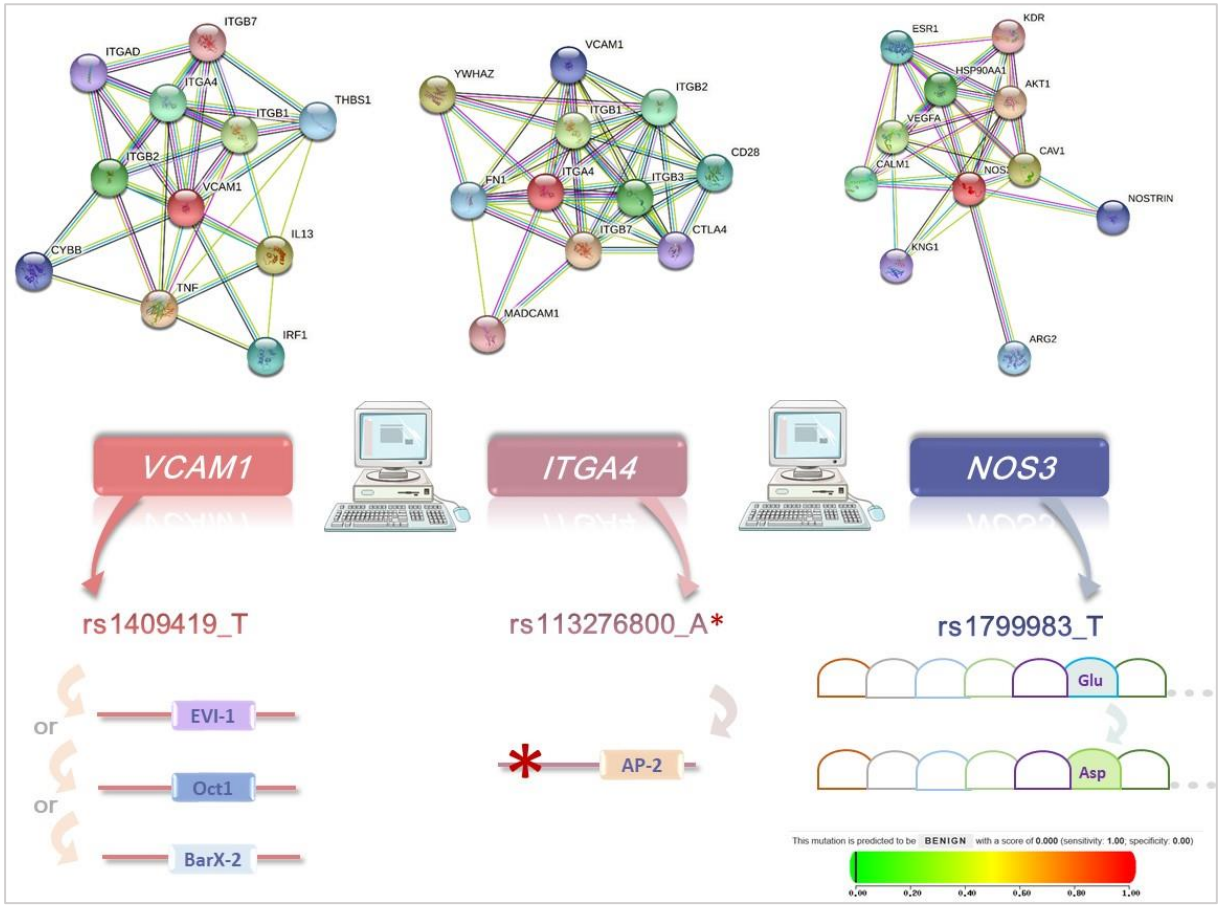
*sources: Ensembl, dbSNP; **total number of patients with genotype results ***1000Genomes – African Yoruba; **Chr** – chromosome; **MAF** – minor allele frequency;

Chapter 3

HEMORHEOLOGICAL ALTERATIONS IN SICKLE CELL ANEMIA AND THEIR CLINICAL CONSEQUENCES

The Role of Genetic Modulators

Graphical abstract



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3.1. SUMMARY

This work aimed to characterize, by *in silico* studies, the role of specific genetic variants in modulating severe hemolysis and stroke risk in children with SCA, as well as understand their consequences at the hemorheological level. Association studies were performed between hemolysis biomarkers and the degree of cerebral vasculopathy additionally to the inheritance of several polymorphic regions in vascular adhesion molecule-1 (*VCAM1*), α4 integrin (*ITGA4*), and endothelial nitric oxide synthase (*NOS3*) genes related to cell adhesion and vascular tone in pediatric SCA patients. *In silico* tools (e.g. *MatInspector*, *TFbind*) were applied to investigate the potential pathophysiological consequences. Variants in the *VCAM1* promoter and *ITGA4* were previously associated with stroke and/or severe hemolysis. Promoter variants, like the *VCAM1* rs1409419_T allele, potentially modify transcription factor binding sites while nonsynonymous variants, like *NOS3* rs1799983_T, disturb the corresponding protein structure/function. Our findings emphasize the relevance of genetic variation in modulating the disease severity due to their effect on gene expression or modification of protein biological activities related to sickled RBC-endothelium interactions and consequent hemorheological abnormalities.

Keywords: Sickle cell anemia, *VCAM1*, *ITGA4*, *NOS3*, genetic modulators, *in silico* analysis

3.2. BACKGROUND

The pathogenesis of SCA primarily derives from the polymerization of deoxygenated HbS inside RBCs which, in turn, leads to their distortion and sickling, reduced deformability, increased vascular adhesion, and ultimately to cell death and hemolysis [378–380]. The disease presents with high clinical heterogeneity characterized by chronic hemolysis, recurrent painful episodes of severe vaso-occlusion, and infection.

A part of hemolysis-associated free-Hb quickly reacts with haptoglobin, forming a complex that is cleared from plasma. However, due to the elevated rate of hemolysis in SCA, the excess cell free-Hb scavenges NO thus decreasing NO bioavailability. The decline in blood NO concentration leads to endothelial dysfunction, over-expression of vascular adhesion molecules, and impaired vasomotor tone [165].

As discussed in chapter 1, vascular occlusion and hemolytic anemia, are leading underlying mechanisms of the SCA clinical complications, due to cumulative multi-organ damage and ultimately a shortened lifespan. Vaso-occlusion is thus believed to occur as a multi-step process that involves interactions between sickle RBCs, activated leukocytes, ECs, platelets, and plasma proteins [378]. Recurrent vaso-occlusion, as well as ischemia-reperfusion, with consequent vascular endothelial activation and injury, induce a continuous inflammatory response in SCA individuals that is propagated by the release of high levels of inflammatory cytokines, decreased NO bioavailability, and oxidative stress [378]. The ECs, activated by cytokines and low NO bioavailability, may provide the basis for decreased vasodilation, blood cell adhesion, and micro-thrombosis in specific organs, like the lung. As stated earlier, ECs have different properties in the vascular beds of different organs. The organs where vasculopathy occurs more frequently in SCA may potentially be the most dependent on NO bioactivity for normal function [381].

The long-discussed phenotypic heterogeneity and multifactorial-like behavior of SCA have prompted the hypotheses that this single gene disorder is under polygenic control, i.e. severity modulation by genetic modifiers. In altered EC function, for instance, one of the putative modifier genes is *VCAM1* which encodes the vascular cell adhesion molecule-1 (VCAM-1), a cytokine-inducible cell surface glycoprotein present in ECs in inflammatory conditions. Through its ligand, α4 integrin, whose gene is another potential modifier, it mediates monocyte and leukocyte adhesion to the endothelium, especially in small vessels [378]. In SCA, it promotes sickle RBCs endothelial adhesion and, therefore, vaso-occlusion [381]. *NOS3*, the eNOS gene, is another likely SCA genetic modulator known to be involved in vascular homeostasis. Contrary to VCAM-1, eNOS is a constitutive enzyme responsible for NO endothelial production, fundamental for the vasoconstriction/vasodilation balance due to its role in smooth muscle cell relaxation [163].

In silico methods relying on bioinformatics tools may be used to provide preliminary information on the genetic variants identified and their putative structural/functional consequences when compared with the wild-type genome sequences. Several of these research tools are available for analyzing variant sequences, fundamentally relying on the type of sequence change to be analyzed, and on the algorithm and databases applied by the different tools. For regulatory genomic regions, such as gene promoters, capable of predicting which changes may lead to modified gene expression levels may provide useful information on gene regulation impairment. Analysis of putative transcription factor binding sites constitutes a way to achieve that end and relies on assessing the degree of similarity between a given sequence and the consensus sequence corresponding to a specific transcription factor. As for coding regions, the prediction of both potential and functional changes is based on the

comparison with the wild-type protein structure. Non-synonymous variants (i.e., associated with amino acid change) are often pathogenic, while synonymous variants (i.e., not associated with amino acid change) are more likely to be benign.

In the present study, *in silico* tools were applied for analysis and characterization of the structure and function of *VCAM1* promoter and *ITGA4* variants associated with hemolysis severity [354] and stroke in SCA pediatric patients [382], as described in the previous chapter. An emphasis is given to *VCAM1* rs1409419_T due to its potential as a standalone modifier. *VCAM1* rs1041163_C and rs3917025_delCT were also included for comparison purposes since, as discussed in chapter 2, the modifier potential of these and the other described *VCAM1* promoter variants would be more correctly assessed in the context of the haplotypes that contain them. The nonsynonymous *NOS3* rs1799983_T variant associated with cardiovascular risk [383] and with SCI protective haplotypes [382] was also analyzed.

3.3. METHODOLOGY

An initial characterization of the three molecules, VCAM-1, ITGA4 (α4 integrin), and eNOS, was performed using the following databases, described in Table 3.1: Molecular Modeling Database (MMDB, https://www.ncbi.nlm.nih.gov/Structure/MMDB/mmdb.shtml), Protein Data Bank (RCSB PDB, https://www.rcsb.org), Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.genome.jp/kegg), Gene Ontology (GO, http://geneontology.org) and the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING, https://string-db.org).

To evaluate possible transcription factor binding site changes that might influence *VCAM1* gene expression regulation, the wild-type sequence and three variants

(rs1409419_T, and rs1041163_C and rs3917025_delCT) within the gene promoter region were compared *in silico*. The nucleotide sequence of the core *VCAM1* promoter and their variants were obtained from the NCBI (http://ncbi.nlm.nih.gov) and ENSEMBL (http://www.ensembl.org) databases, spanning from –2180 to +101 bp relative to the main transcription starting site. The MatInspector (www.genomatix.de) [384] tool was used for the transcription factor binding sites analyses (Table 3.2), with a threshold of 0.85 (which corresponds to a maximum of 15% dissimilarity between the identified sequence and the consensus sequence). The following transcription factors were considered in particular, due to their role in inflammation, cell proliferation, and oxidative stress: NF-κB, ETS transcription factor family, GATA transcription factor family, TATA box binding factors, specificity protein 1 transcription factor (Sp1), octamer-binding transcription factor (Oct-1), and EVI-1. The putative transcription factor binding sites resulting from the *in silico* analysis of the SNPs were cross-referenced with the experimentally validated sites described previously by [124] and [107]. The Variant Effect Predictor (from the ENSEMBL search engine) was also used to assess potential functional consequences of the *VCAM1* promoter variants. The putative functional consequences of the nonsynonymous variant of *NOS3* (rs1799983_T) were analyzed through the use of the following *in silico* tools specific for evaluating missense mutations effects on protein function: Sorting Intolerant from Tolerant (SIFT) [385], Polyphen 2 [386], Domain Mapping of Disease Mutations (DMDM) and PredictSNP [387]. The latter combines predictions of eight established prediction tools (MAPP, nsSNPAnalyzer, PANTHER, PhD-SNP, PolyPhen-1, PolyPhen-2, SIFT, and SNAP) and transforms the individual confidence scores to one comparable scale of 0–100%, using the values of their observed accuracies [387]. OMIM (http://ncbi.nlm.nih.gov/omim), ENSEMBL, dbSNP (https://www.ncbi.nlm.nih.gov/snp/)

and ClinVar (http://www.ncbi.nlm.nih.gov/clinvar) databases were also used to assess SNP frequencies and disease association/pathogenicity references.

Table 3.1. Overview of the databases/repositories used in this study

Database	Purpose
OMIM	Comprehensive compendium of human genes and genetic phenotypes.
ClinVar	Archives/aggregates information about relationships among variation and human health
ENSEMBL	Joint project between EMBL-EBI and the Wellcome Trust Sanger Institute to develop a software system that produces and maintains automatic annotation on selected eukaryotic genomes
MMDB	Experimentally resolved structures of proteins, RNA, and DNA, derived from PDB, and added features (e.g., computationally identified 3D domains to identify similar 3D structures)
PDB	Repository of information about the 3D structures of large biological molecules, including proteins and nucleic acids
KEGG	Database resource for understanding high-level functions and utilities of the biological systems, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies
STRING	Database of known and predicted protein interactions, including direct (physical) and indirect (functional) associations, derived from genomic input, high-throughput experiments, co-expression, and previous knowledge

Table 3.2. Overview of the *in silico* prediction tools used for this study

Tool	Type	Purpose	Input	Output
MatInspector	PT	Utilizes a large library of matrix descriptions (IUPAC) for transcription factor binding sites to locate matches in DNA sequences. It assigns a quality rating to matches for filtering and selection of matches.	Nt sequence, accession number, personal DB	Predicted transcription factor binding site
Variant Effect Predictor	PT	Predicts the effect of variants (SNPs, insertions, deletions, CNVs, or structural variants) on genes, transcripts, and protein sequence, as well as regulatory regions	dbSNP id.	Several scores and information according to tools/datasets used
SIFT	PT	Predicts if an AAS affects protein function	AAS, SNP, or protein accession number	Score ranges from 0 to 1; 0=neutral, 1=damaging
PolyPhen2	PT	Predicts the possible structural and functional impact an aaS may have on a human protein using physical and comparative considerations	Nt/AA	Score ranges from 0 to a positive number; 0=neutral; high positive number = damaging
Domain Database for Disease Mutations	DB/PT	Domain mapping of disease mutations (DMDM) is a database in which each disease mutation can be displayed by its gene, protein, or domain location	Gene, AA, domain, SNP accession number	
PredictSNP	PT	Consensus classifier (dataset) that combines six of the top-performing tools for the prediction of the effects of mutation on protein function	AA	Probabilities (%) provided together with annotations extracted from PMD and the UniProt database.

PT – prediction tool; **DB** – database; **Nt** – nucleotide; **SNP** – single nucleotide polymorphism; **CNV** – copy number variant; **AA** – amino acid; **AAS** – amino acid substitution; **id** – identification; **PMD** – Protein Mutant Database

3.4. RESULTS

3.4.1. VCAM-1 Characterization

As mentioned above, VCAM-1 is a 110 kDa highly conserved immunoglobulin-like transmembrane adhesion molecule first identified as being located on the surface of ECs in the process of inflammation and immune response [108] (Fig. 3.1). It is a homodimeric protein encoded by the *VCAM1* gene, located on chromosome 1q31-q32, spanning 25kb and encompassing 9 exons. Besides EC expression, it has also been found to be expressed by various cell types including stromal bone marrow, skeletal muscle, and different hematopoietic cell types [107,124]. ECs from both large and small blood vessels are known to highly express cell surface VCAM-1 following cytokine stimulation and sickle erythrocytes were found to have a propensity to adhere to VCAM-1 via the very late antigen-4 (VLA-4 or α4β1) expressed on their surface membrane [381]. Like other members of the cell adhesion molecules (CAMs), VCAM-1 is cytokine-inducible and interacts with several functional partners (Figs. 3.2 and 3.3), such as integrins, constituting part of the mechanisms through which inflammatory cells can be recruited to the endothelium [124]. There is evidence that, as other endothelial cell ligands, it is involved not only in cell-cell interactions but also in nuclear signaling and tumor cell metastasis [107,124]. Additionally, it is known that, in lymphoid tissue, VCAM-1 expression is constitutive – not cytokine-dependent, as in endothelial cells – and mediates the interaction of maturing B-cells with dendritic cells at the germinal center [124]. Therefore, different patterns of expression in different tissues suggest that the *VCAM1* gene is subjected to alternate regulatory mechanisms in different tissues as reported by Iademarco *et al* [124].

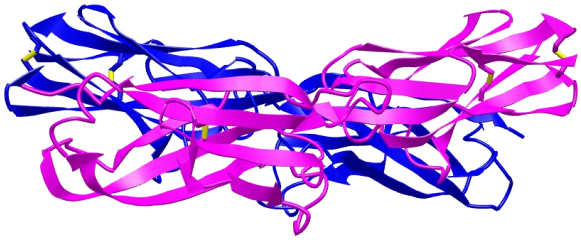

Figure 3.1. ***VCAM-1 protein structure.*** 3D structure obtained from the Molecular Modelling Database (ID: 4464) [388] and Protein Data Bank repository (ID: 1VSC), using the iCn3D software[7].

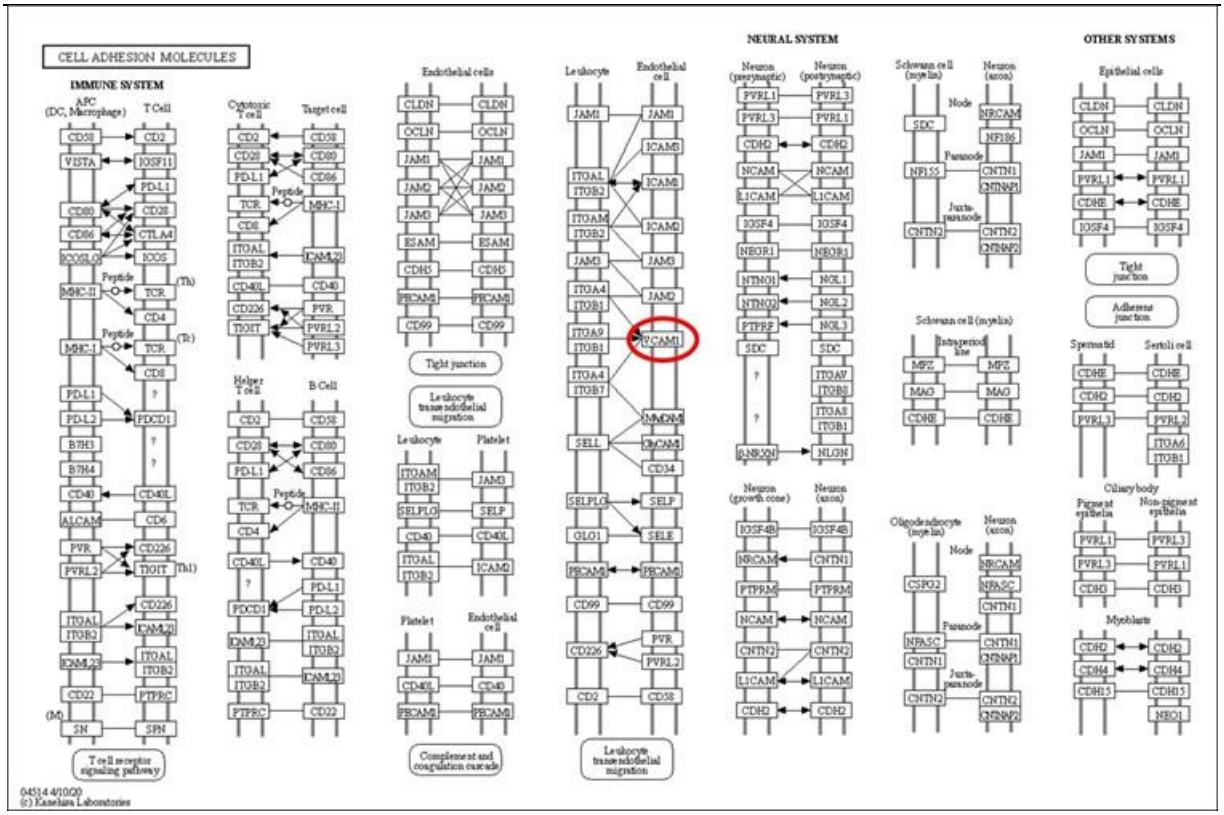

Figure 3.2. ***VCAM-1 functional positioning and interactions in cell adhesion*** (highlighted in a red circle). Retrieved using the KEGG Genome database, entry 7412.

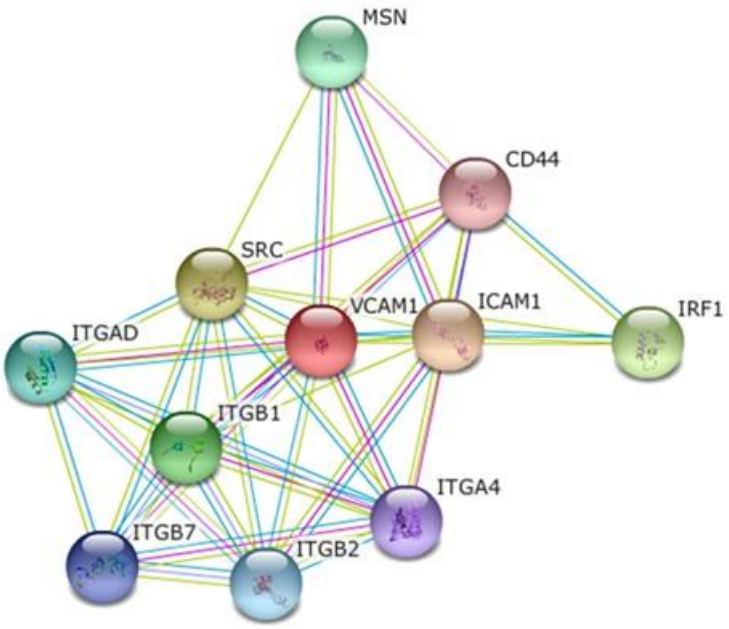

Figure 3.3. ***VCAM-1 predicted functional partners and interactions.*** ICAM1, intercellular adhesion molecule 1; SRC, v-src sarcoma; ITGB1, integrin beta 1; ITGA4, integrin alpha 4; ITGAD, integrin alpha D; MSN, moesin; CD44, CD44 molecule; ITGB2, integrin beta 2; IRF1, interferon regulator factor; ITGB7, integrin beta 7. Retrieved using STRING tool.

Characterization of the structural elements of the *VCAM1* promoter has provided important insights into the induced expression of this adhesion molecule and by inference into events underlying several pathophysiological mechanisms [108,124]. The *VCAM1* promoter nucleotide (nt) sequence has been previously established as -2180 to +101 bp relative to the main transcription start site (TSS) region (Fig. 3.4) and eight key transcriptional binding sequences have been identified: TATA box, NF-κB, AP1, ETS, GATA, Oct, TEF-1, and consensus binding sites for transcription factors across the 5' region (Tab. 3.3). AP1, NF-κB, and ETS sites are known to be involved in cell cycle control and can provide targets for constitutive activation of the *VCAM1* promoter. GATA, ETS and Oct binding proteins are all critical for development and additionally, GATA1 and ETS1 may be important in endothelium differentiation. The presence of

binding sites for development-specific proteins in the *VCAM1* promoter probably indicates a role for VCAM-1 in development as occurs in muscle differentiation [110].

The possibility that genetic variability of *VCAM1* might lead to a disease modulation role due to transcription level changes, and hence in this adhesion molecule function, has been evaluated [343,354,355].

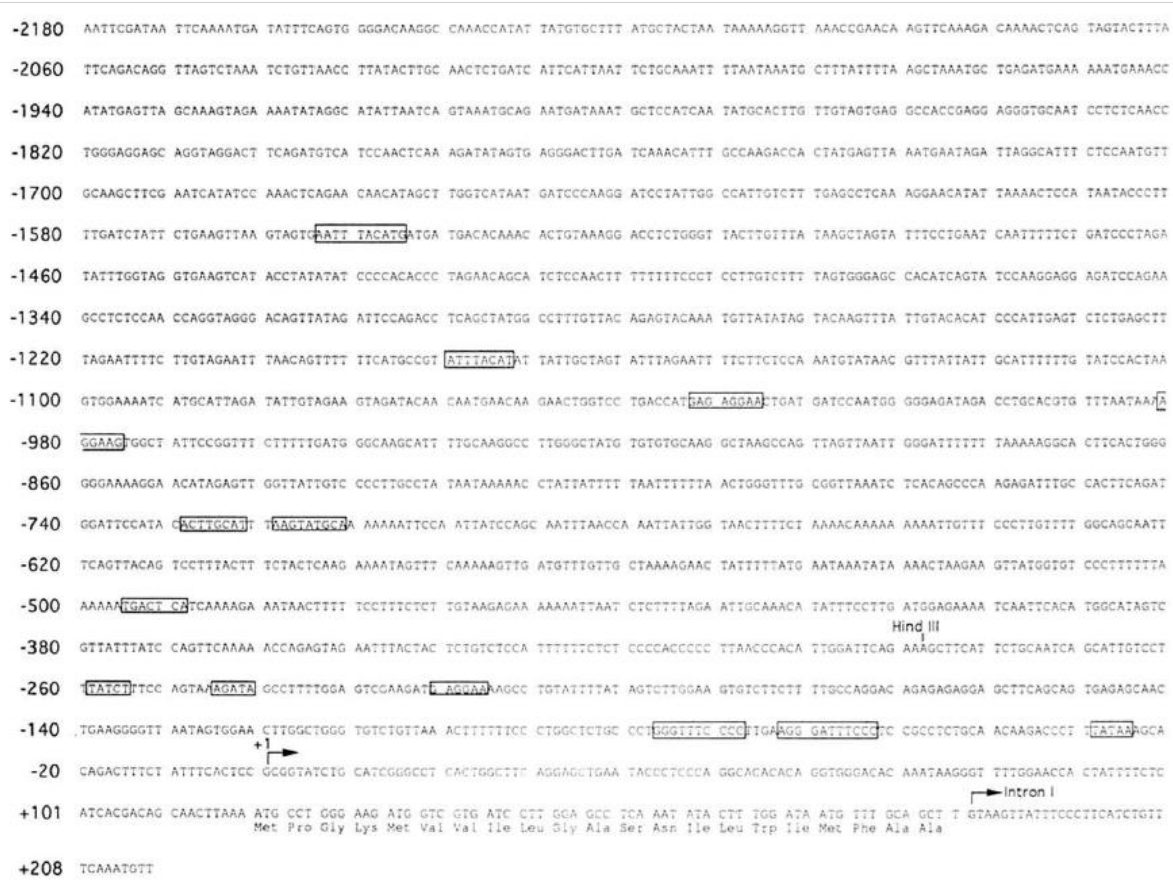

Figure 3.4. *VCAM1* sequence of the 5' end and flanking region. The transcriptional initiation site is indicated by an arrow and +1. Potential regulatory elements are highlighted in boxes and further discussed in the text and table 3.3. Reproduced from [124], by permission of *The Journal of Biochemistry Journal,* under a CC-BY-NC-ND license.

Table 3.3. Characterized transcription factors binding sites in human *VCAM1* gene promoter

Transcription factor	Position(s) (bp)	Function
TATA	-29	Indicates initiation of transcription, defines the direction of transcription and what strand to be read; absent in genes encoding constitutively expressed molecules
NFkB	-63 -77	Responsive to TNF-α
AP-1	-495	Responsive to TNF-α
ETS	-221 -981 -1033	Involved in cell cycle control by controlling tissue and developmental specific expression
GATA	-259 -245	Binds a family of zinc-finger nuclear proteins that differ in tissue specificity – GATA1 is involved in red cell differentiation; GATA2 in endothelin-1 expression (important in endothelial cell differentiation); and GATA3 is T-cell specific
Oct	-729 -1180 -1554	Binds a family of homeobox proteins differentially expressed in adult tissues during development
TEF1	-719	Binds GT-II C, Sph-I, Sph-II enhancers in the SV40 enhancer

3.4.2. α4 Integrin Characterization

VLA-4 is the only integrin maintained on the surface of young sickle RBCs and reticulocytes, which are increased in the peripheral blood of SCA patients. It is an atypical very-late antigen that mediates sickle RBC adhesion to the endothelium via VCAM-1 and to the ECM via fibronectin [389]. VLA-4 is a heterodimeric integral membrane protein, with one α and one β subunit, that recognizes the Q-I-D-S sequence in VCAM-1. *ITGA4* (chromosome 2q31.3) encodes the α subunit which associates with a β1 unit forming the α4β1 integrin or VLA-4.

Initially, a pre-protein is formed that after proteolytic processing generates light and heavy chains that compose the α4 subunit (Fig. 3.5). Alternative splicing may occur, giving rise to multiple transcript variants. With 114.9 kDa and 1032 amino acids, this subunit may be cleaved into two non-covalently associated fragments. When associated with a β1 subunit, forming VLA-4 it is involved homotypic leukocyte aggregation, leukocyte-endothelium, and ECM adhesion, as well as transendothelial migration (Figs.3.6, 3.7, and 3.8).

In SCA, VLA-4 mediates the formation of aggregates of sickle RBC and circulating leukocytes promoted by fibronectin bridging of monocytes and reticulocytes via their surface VLA-4 during flow conditions [390,391]. These sickle RBC-leukocyte aggregates contribute to vascular occlusion in SCA [236] and lead to local hypoxia, increased HbS polymerization, and further vessel obstruction.

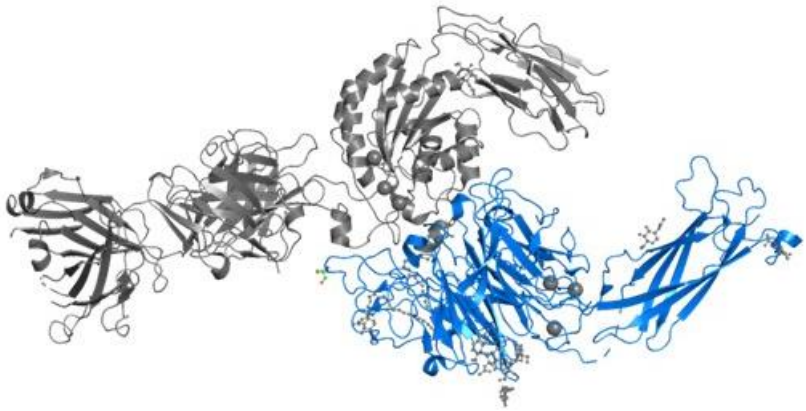

Figure 3.5. ***α4 integrin subunit.*** Depicted here in blue (front view), associated with β7 subunit complexed with Fab ACT-1 (grey). 3D structure obtained from the Protein Data Bank Europe repository (PDBe ID:3v4p), using the iCn3D software [7].

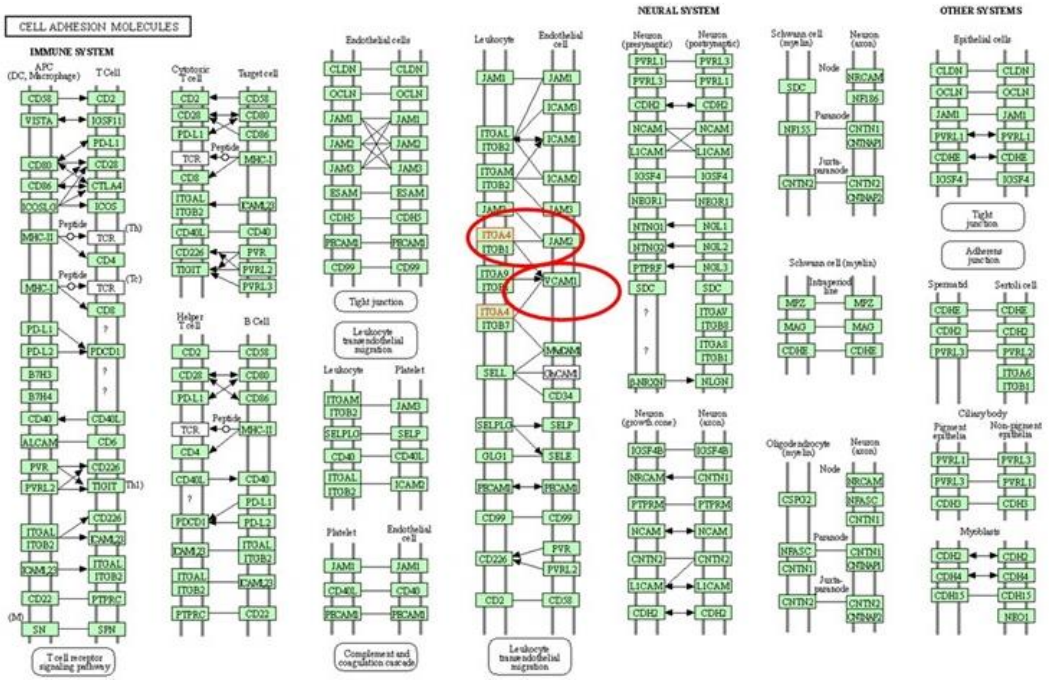

Figure 3.6. ITGA4 functional positioning and interactions in cell adhesion. Main interactions highlighted in red circles. Retrieved using the KEGG Genome database, entry 3676.

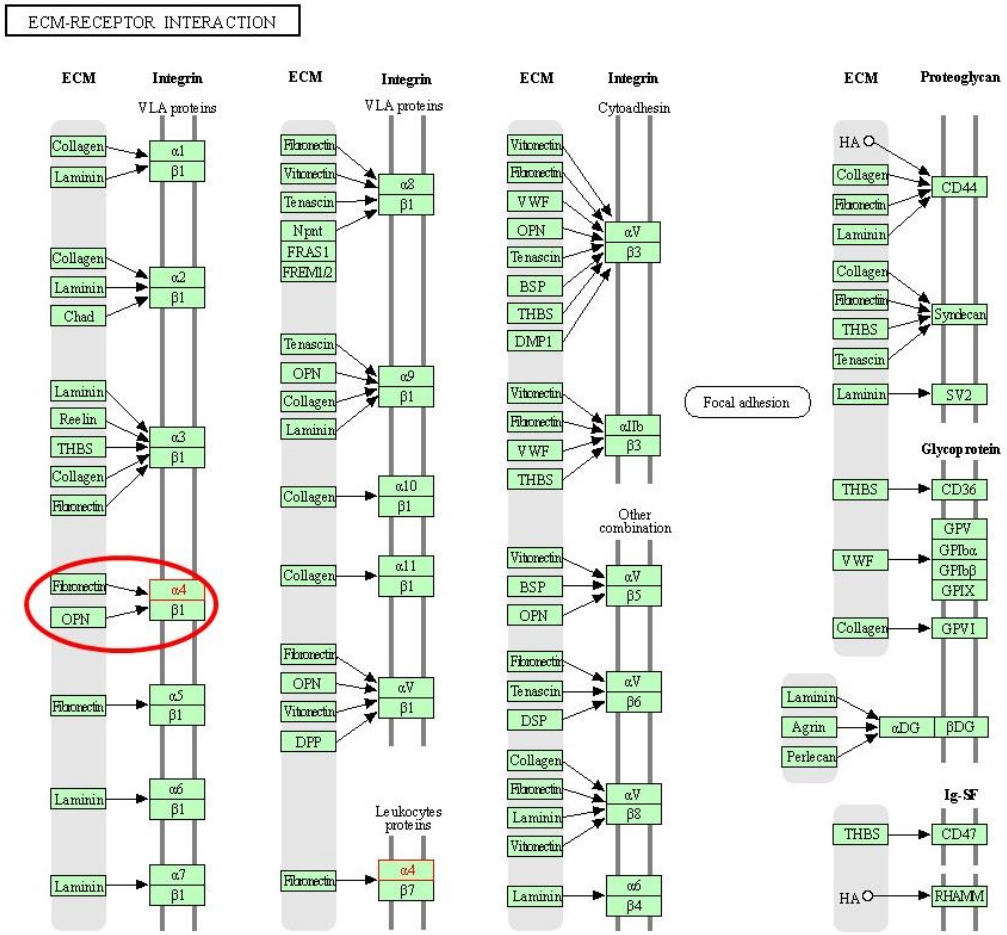

Figure 3.7. *α4β1 integrin functional positioning in endothelial cell matrix (ECM)-receptor interactions.* Main interaction is highlighted with a red circle. Retrieved using the KEGG Genome database, entry 3676.

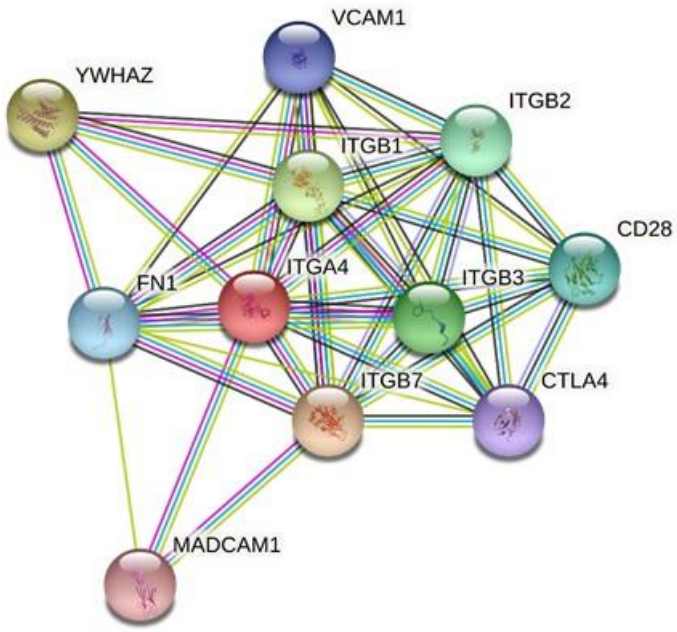

Figure 3.8. ***ITGA4 predicted functional partners and interactions.*** VCAM1, vascular cell adhesion molecule 1; FN, fibronectin 1; MADCAM1, Mucosal addressin cell adhesion molecule 1; YWHAZ, 14-3-3 protein zeta/delta; ITGB1, integrin beta 1; ITGB2, integrin beta 2; ITGB3, integrin beta 3; ITGB7, integrin beta 7; CD28, T-cell-specific surface glycoprotein CD28; CTLA4, Cytotoxic T-lymphocyte protein 4. Retrieved using the STRING tool.

3.4.3. eNOS Characterization

The eNOS, a 130 kDa protein encoded by the *NOS3* gene, is one of three NO generating isoforms of the enzyme NO synthase and has a regulatory function in the cardiovascular system [78]. The gene encompasses 26 exons throughout 21 kb and, like *VCAM1*, is mainly expressed in ECs but has also been detected in other cell types such as cardiac myocytes, platelets, in certain neurons of the brain, syncytiotrophoblasts of the human placenta, and in LLC-PK1 kidney tubular epithelial cells (reviewed in [78]). However, unlike VCAM-1, it is expressed constitutively and is crucial for the vasoconstriction/vasodilation balance that is of the utmost importance in vascular homeostasis and therefore in the overall vascular health (Fig. 3.9).

This heme-containing protein catalyzes the five-electron oxidation of the guanidine nitrogen of L-arginine (substrate) to NO and citrulline, through a process that requires oxygen and reduced nicotinamide-adenine-dinucleotide phosphate (NADPH) as co-substrates and several co-factors such as calcium, calmodulin, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and tetrahydrobiopterin (BH_4) [78,392] (Fig. 4). The functional eNOS is a homodimer that transfers electrons from NADPH, via FAD and FMN in the carboxy-terminal reductase domain, to the heme in the amino-terminal oxygenase domain [78]. The oxygenase domain also binds the essential cofactor BH_4, molecular oxygen, and the substrate L-arginine. At the heme site, the electrons are used to reduce and activate O2 and to oxidize L-arginine to L-citrulline and NO. Sequences located near the cysteine ligand of the heme are also apparently involved in L-arginine and BH_4 binding. eNOS synthesizes NO through two steps: (i) first by hydroxylating L-arginine to N^{ω}-hydroxy-L-arginine which remains largely bound to the enzyme and (ii) second by oxidizing N^{ω}-hydroxy-L-arginine to L-citrulline and NO. eNOS, as all isoforms of NOS, binds calmodulin, which is brought about by an increase in intracellular Ca^{2+} and facilitates the flow of electrons from NADPH in the reductase domain to the heme in the oxygenase domain. However, eNOS may also be activated through mechanisms that do not lead to intracellular Ca^{2+} increases, such as phosphorylation-mediated fluid shear stress [78].

The main physiological functions of eNOS involve several functional partners (Fig. 3.9) and include vasodilation and inhibition of platelet aggregation and adhesion, inhibition of leukocyte adhesion and vascular inflammation, control of vascular smooth muscle proliferation, stimulation of angiogenesis by eNOS-derived NO and, activation of endothelial progenitor cells by eNOS-derived NO [78]. The NO formed by the NOSs can act as a signaling molecule to several target enzymes and proteins, namely in the activation of soluble guanylyl cyclase and generation of cyclic GMP (reviewed in [78]).

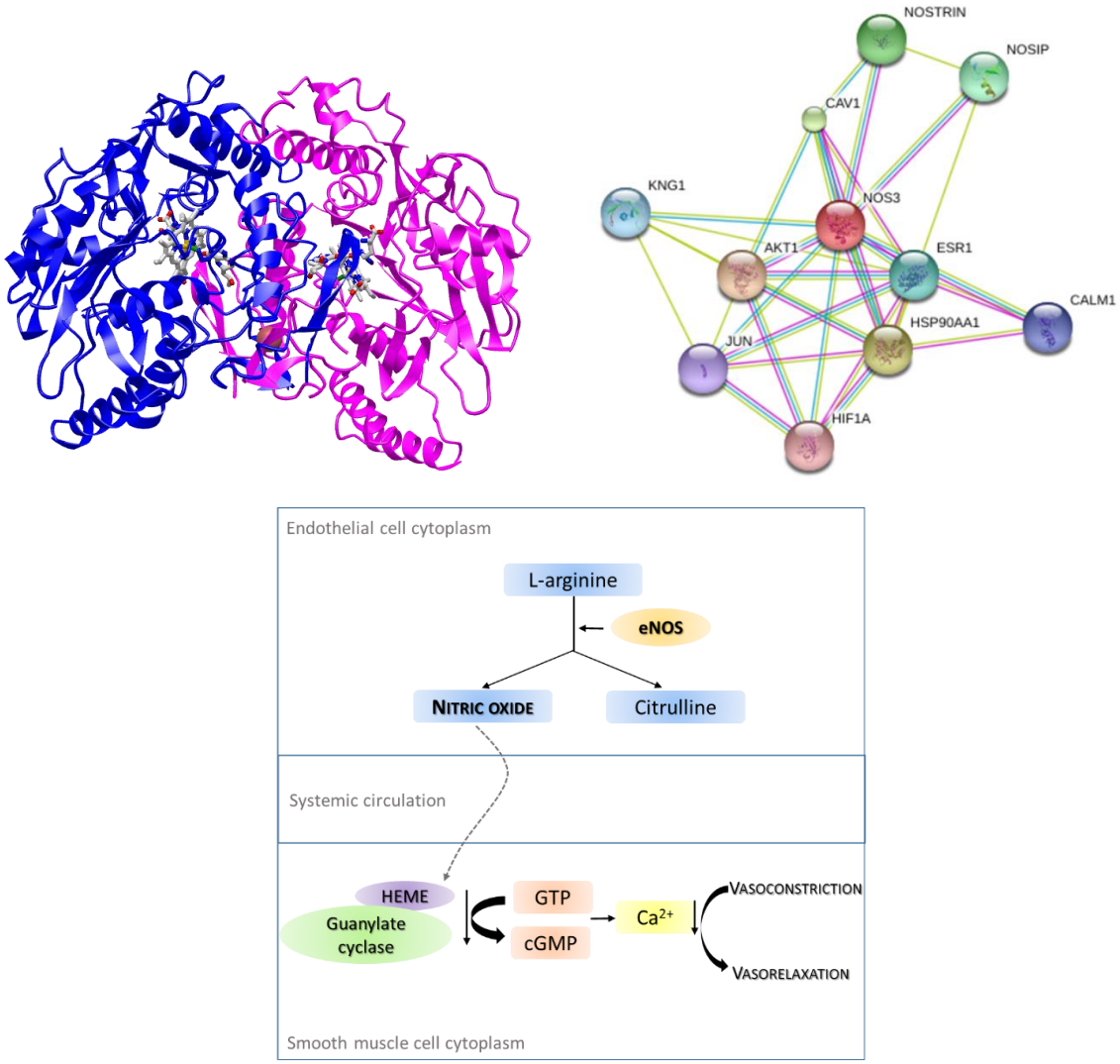

Figure 3.9. ***eNOS structure and main functions.*** Top left: 3D structure, obtained from the Protein Data Bank repository (ID: 3NOS) [393], using the iCn3D software [7]. Top right: predicted functional partners and interactions (Retrieved using STRING Tool). AKT, v-akt murine thymoma viral oncogene homolog 1; CAV, caveolin 1; ESR1, estrogen receptor 1; CALM1, calmodulin; HSP90AA1, heat shock protein 90; HIF1A, hypoxia-inducible factor 1; JUN, jun protooncogene; KNG, kininogen 1; NOSTRIN, nitric oxide synthase trafficker; NOSIP, nitric oxide synthase interacting protein. Bottom left: NO production from L-arginine, leading to vasodilation.

Although there is no evidence that eNOS is a 'disease gene', many cardiovascular risk factors lead to oxidative stress, eNOS uncoupling, and endothelial dysfunction in the vasculature. The G894T missense mutation (rs1799983_T) occurring in exon 7 of

NOS3 has been described previously in association with increased susceptibility for cardiovascular disease and with a frequency of 35 – 78% was considered a coding polymorphism [394–396]. It has been hypothesized that it might lead to modifications in the original caveolar localization of eNOS, although not in the substrate binding of this molecule [396].

3.4.4. *In Silico* Analysis of *VCAM1* Promoter Variants

Our group has previously performed an association study between hemolysis biomarkers (serum LDH, total bilirubin, and reticulocyte count) and the inheritance of genetic variants of ten candidate genes related to vascular tonus, vascular cell adhesion, inflammation, fetal hemoglobin expression, and alpha-thalassemia, in a series of 99 pediatric SCA patients [354]. Subsequently, and as described in the previous chapter, we have performed association studies evaluating the role of the same genetic variants, as well as of hematological and biochemical parameters, in pediatric cerebral vasculopathy modulation. In both studies, variants in a gene related to adhesion of sickle RBCs to vascular endothelium (*VCAM1* rs1409419_T, rs3917025_delCT, and rs1041163_C) were associated with the SCA hemolysis severity and/or with stroke/stroke_risk. On the other hand, one *NOS3* gene variant (rs1799983_T) implicated in cardiovascular risk was also analyzed [383]. Thus, potential consequences of those genetic variants were evaluated *in silico* prior to in vitro functional studies. When the three non-coding variants of *VCAM1* were searched in ClinVar and Variant Effect Predictor (ENSEMBL), two common features emerged: (i) to date, they are considered upstream gene variants, and (ii) their probable functional impact would be as modifiers. The transcription factor binding site analyses performed with MatInspector (Table 3.5) revealed several potential effects. The input data format used

was the SNP database identification number, with the analysis results corresponding to the minor frequency allele. For rs1041163, the T>C change led to a potential substitution of an RXRF by a PRDM1 binding site and the potential loss of an FHXB binding site. The presence of the rs1409419_T allele leads to a possible gain of several homeobox transcription factor binding sites, in particular, for EVI-1, Oct1, and BarX2. Regarding rs3917025_del CT, a potential gain of a FAST1 binding site was indicated.

Table 3.4. *VCAM1* promoter variants' sequences

Variant ID	Sequence
rs1041163	AAGCTAGTATTTCCTGAATCAATTT[C/T]TCTGATCCCTAGATATTTGGTAGGT
rs1409419	AAAGTAGAAAATATAGGCATATTAA[C/T]CAGTAAATGCAGAATGATAAATGCT
rs3917025	AGGCCACCGAGGAGGGTGCAATCCT[delCT/CT]CAACCTGGGAGGAGCAGGTAGGACT

Sequences were obtained using NCBI's SNP search engine, https://www.ncbi.nlm.nih.gov/snp/

Table 3.5. Transcription factor binding site analysis of *VCAM1* promoter variants

Variant ID	Transcription factor change	Position	Transcription factor binding site sequence
rs1041163_C	RXRF→PRDM1 ('substitution') FHXB ('loss')	-1592	gatcagaGAAAttgattca ~~gggatcAGAAaaattga~~
rs1409419_T	EVI1; Oct1; BarX2 ('gain') *	-2021	tagaaaaTATAggcata ggCATAttaatcagt ggcatatTAATcagtaaat
rs3917025_delCT	FAST1 ('gain')	-1944	caggttgagGATTgcac

*potential gain of several other homeobox genes

3.4.5. *In Silico* Analysis of *ITGA4* Variants

The rs113276800_A and rs3770138_T SNPs, described in the previous chapter to show a positive association with pediatric ischemic stroke, were part of 32 validated *ITGA4* variants identified through NGS.

The transcription factor binding site analysis did not show an overlap of rs113276800_A with sequences for binding sites of critical transcription factors. Therefore, no severe effects on *ITGA4* expression are predicted due to the presence of this variant.

Table 3.6. *ITGA4* promoter variant rs113276800 possible sequences

SNP	(Alternative) Sequences
rs113276800	GCCCGAACGCTCCGCCCGCGGTGGG**[C]**CGACTTCCCCTCCTCTTCCCTCTCT
	GCCCGAACGCTCCGCCCGCGGTGGG**[A]**CGACTTCCCCTCCTCTTCCCTCTCT
	GCCCGAACGCTCCGCCCGCGGTGGG**[T]**CGACTTCCCCTCCTCTTCCCTCTCT

Sequences were obtained using NCBI's SNP search engine, http://ncbi.nlm.gov/snp

The sequence of rs113276800_A was not found to overlap with any of the previously reported transcription factor binding sites consensus sequences for the *ITGA4* promoter. Moreover, at the 0.85 threshold which corresponds to a maximum of 15% dissimilarity between the identified sequence and the consensus sequence, we did not find any significant changes resulting from the presence of this variant. Nevertheless, as stated in the previous chapter, rs113276800_A is located near the AP-2 binding sites of the *ITGA4* promoter region, which in homozygosity would potentially cause a negative expression of the integrin α4 subunit [368].

The rs3770138_T variant is classified in the ENSEMBL database as an intron variant or a transcript variant of a non-coding RNA gene. Furthermore, analysis with Human

Splicing Finder (v. 3.1) did not reveal potentially altered splicing sites due to the presence of this variant. Therefore, no direct effects were predicted to be associated with the presence of this variant.

3.4.6. *In Silico* Analysis of *NOS3* Missense rs1799983_T Variant

The DMDM analysis indicated that the nonsynonymous variant p.Glu298Asp is located in the oxygenase domain-coding region of *NOS3*. This tool is linked to the OMIM database which associates this genetic variant to susceptibility for coronary heart spasm, late-onset Alzheimer's disease, and hypertension (Fig. 3.10). These results were consistent with the ones obtained when assessing the ClinVar database and arise from taking into account all reports of the presence of the genetic variant in association with a given phenotype. For the analysis of the putative functional consequences three bioinformatics tools were used: SIFT, PolyPhen 2, and PredictSNP. These tools predict the consequences of mutations that are translated into amino acid changes in the protein structure, based on algorithms and comparison with known disease variation databases. The SIFT results indicated that this variant is tolerated. The PolyPhen 2 results were consistent with the SIFT ones, with a benign classification for this variant whether considering HumDiv or the HumVar databases' references (Fig. 3.11). The non-pathogenic category was also attributed by the combined PredictSNP tool results (83%) which are based on analyses performed by five different tools – SIFT (71%), PolyPhen 2 (74%), PolyPhen 1 (67%), SNAP (71%) and PhDSNP (78%) (Fig. 3.12). Nevertheless, in this case, a possible association with cardiovascular disease susceptibility was considered, which is in accordance with the above-mentioned databases' assessment.

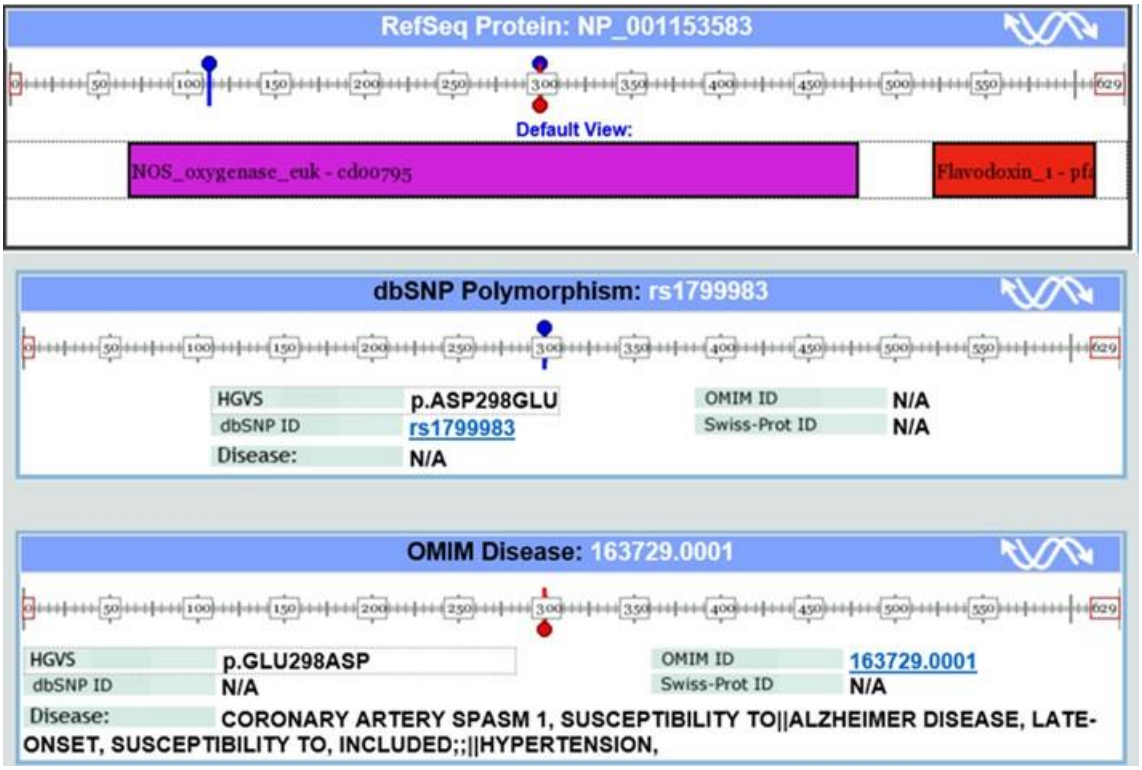

Figure 3.10. ***Results of the analysis of the NOS3 rs1799983_T variant, using the*** **Domain of Mapping of Disease Mutations** ***(DMDM) database***. The location of the mutation in the oxygenase domain of eNOS is shown, as well as the classification of Online Mendelian Inheritance in Man (OMIM) as a susceptibility factor for several diseases with a vascular pathology background.

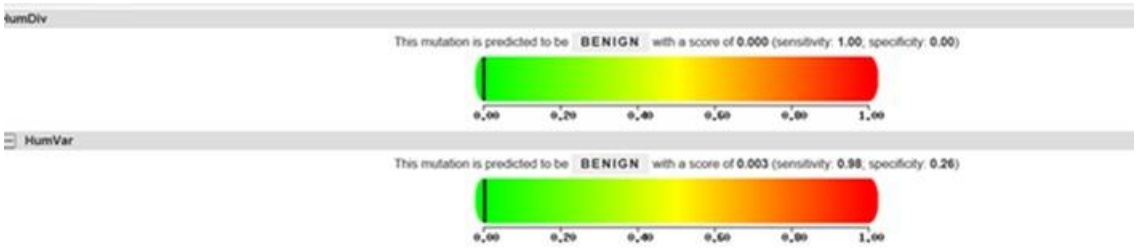

Figure 3.11. ***Results of the PolyPhen2 analysis of NOS3 rs1799983_T variant.*** A potentially benign consequence of the mutation is indicated both by HumDiv (top) and HumVar (bottom).

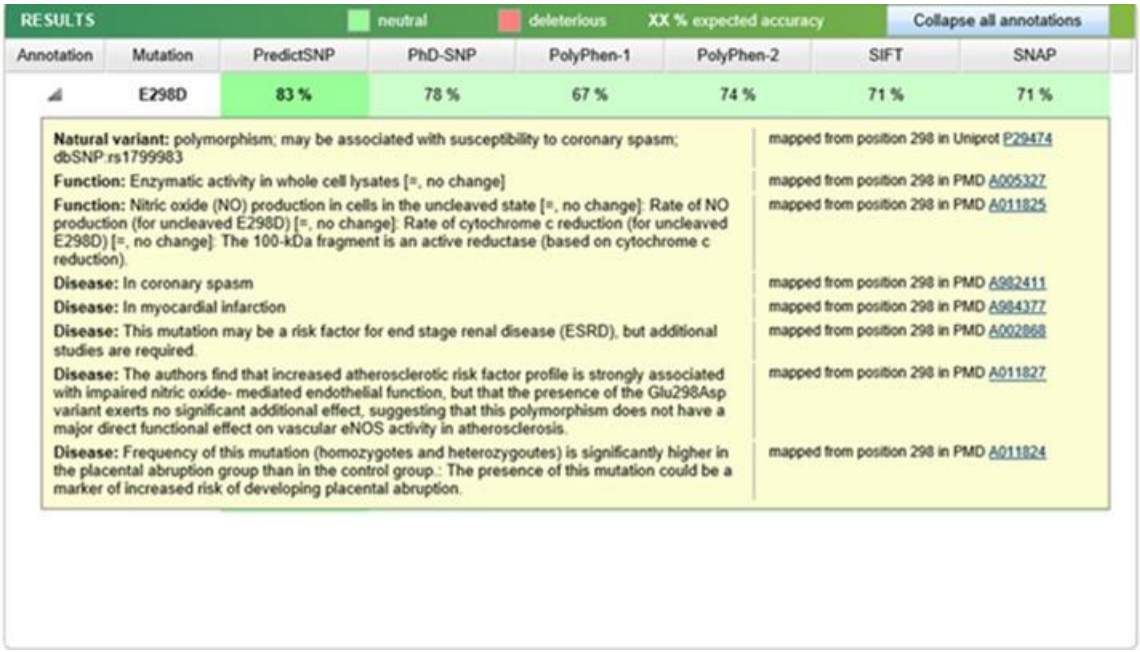

Figure 3.12. ***Results of the PredictSNP analysis of the NOS3 rs1799983_T variant***. A neutral effect of the mutation is indicated, despite an annotation for disease susceptibility/risk in several vascular pathology disorders.

3.5. DISCUSSION

Although the major genetic modifiers of SCA clinical manifestations are those affecting the HbF expression, *VCAM1* and *NOS3* variants have also been identified as potential modulators of the disease. In this study, the results of an *in silico* analysis of *VCAM1* rs1041163_C, rs1409419_T, and rs3917025_delCT noncoding polymorphisms, as well as *NOS3* coding SNP rs1799983_T, provide some clues about possible functional roles of these genetic variants in the pathophysiology of SCA. The three *VCAM1* promoter variants mentioned above have in common the potential for affecting this gene's expression regulation. This may occur as a result of differences in transcription factor affinity to the altered sequence as compared to the wild-type sequence.

In the present work, transcription factor binding site changes were indeed observed for the three polymorphic regions.

Concerning the rs1041163_C, an RXRF by PRDM1 substitution as well as a loss of an FHXB were indicated. PRDM1 is a transcription repressor that promotes differentiation of hematopoietic B cells and secretion of pro-inflammatory cytokines [397]. Therefore, in a pro-inflammatory environment, such as an activated endothelium, this variant might lead to an increase of *VCAM1* inducible expression. In the case of rs1409419 C>T, it was shown to have a potential gain for EVI1, Oct1, and BarX2. EVI1 is a complex multifunctional that modulates multiple processes, including cell migration, motility, adhesion, response to oxidative stress, proliferation, and apoptosis/survival [364]. It contains a GATA consensus motif and prevents DNA binding by GATA1, thus limiting red blood cell differentiation and proliferation [398]. EVI1 has been reported to cooperate with a FOS transcription factor to limit cell adhesion while enhancing cell proliferation, one hallmark of oncogenesis [364]. On the other hand, Oct1 is a transcription factor known to promote a transcriptional repression/silencing effect which would potentially lead to *VCAM1* down-regulation. BarX2 has been shown to promote murine muscle cell differentiation by interacting with muscle regulatory factors [399], a gain of which could result in upregulation of gene expression in muscle tissue. Finally, a gain of a FAST1 binding site was identified for rs3917025_delCT. FAST1 is a transcription factor involved in the patterning and development of embryonic structures in vertebrates, in a complex network of activation/repression mechanisms [400].

In summary, all of the transcription factors affected by the sequence variants are mainly involved in development (including in early embryonic stages, as FAST1) and in different tissues, which is in agreement with the VCAM-1 proposed role in development with tissue- and time-specific expression patterns [110]. In terms of endothelial environment,

for instance, one might expect that altered expression levels may affect sickled erythrocytes/EC adhesion as well as endothelium inflammation/activation, thus contributing to endothelial dysfunction and ultimately to impaired blood flow/shear rate. The *ITGA4* variants may also contribute to altered α4 integrin expression, as they are also regulatory region variants. Even though no direct effect was predicted, we cannot exclude an indirect effect, namely for rs113276800_A due to its proximity to AP-2 transcription factor binding sites. Changes in α4 integrin expression have potential implications in blood cell (including sickle RBCs) adhesion as well as leukocyte transendothelial extravasation and, consequently, in blood flow rate and shear stress.

Regarding *NOS3* rs1799983_T, despite leading to an amino acid change in the protein sequence (p.Glu298Asp), all the analyses showed that this variant is most probably non-deleterious. Therefore, it may be considered a nonsynonymous tolerant SNP. The apparent conservative (negatively charged) amino acid substitution that results therein (aspartate for glutamate) would also be in agreement with that observation. Nevertheless, as the DMDM database results indicate, it occurs in the sequence encoding the oxygenase domain of eNOS, which is critical for the enzyme activity, containing the catalytic site as well as the components of its oxygenase function. Being considered benign, it is reasonable to assume that the variant will probably not affect the main catalytic function. Nonetheless, the oxygenase function may be impaired, thus possibly contributing to higher oxidative stress through decreased heme binding, eNOS uncoupling, and (indirectly) NO bioavailability. Possible alterations in the endothelial location inside the caveolae have also been proposed [396].

Overall, these potentially altered functions play key roles in endothelial dysfunction and/or vascular tone and may modulate SCA severity in terms of cardiovascular risk [401]. Furthermore, impaired oxygenase activity would expectedly result in higher levels

of oxidative stress which (i) has been demonstrated to damage healthy erythrocytes by decreasing their deformability as well as increasing the strength of erythrocyte aggregates, and (ii) have been hypothesized to induce an exaggerated response in erythrocytes from SCA patients, accompanied by a highly abnormal hemorheological profile [reviewed in [402]]. Besides its hemorheological importance, altered oxygenase function may also have an impact on therapeutic approaches. For instance, it is known that drugs interfering with the renin-angiotensin-aldosterone system, as well as statins, are useful in preventing endothelial dysfunction. However, the mechanisms through which they promote eNOS uncoupling, in the case of elevated oxidative stress, may provide useful clues as to ways of increasing NO beneficial actions in the cardiovascular system [78].

Even though *in silico* studies provide information that may prove invaluable for functional experiment managing and planning, they require a careful analysis of specific factors. Concerning input data format, specific sequences already identified in databases provide a lesser margin for error so, using an identification number is in general less prone to error than the manual introduction of a given sequence, for instance. The algorithm that is used for the prediction also determines the tool's accuracy, since it determines sensitivity, specificity and has associated false-positive and false-negative rates. Furthermore, the cut-off values or parameter thresholds are key elements in determining the reliability of results since they are associated with the similarity of the given sequence and a reference sequence and therefore with the likelihood of a specific transcription factor actually binding to that given sequence. Database information, as well as size, quality, and curation also impact a tool's quality and reliability.

Nonetheless, *in silico* approaches only constitute a preliminary step in evaluating genetic variant potential biological and clinical consequences. *In vitro* (and, whenever possible, *in vivo* or *ex vivo*,) studies are crucial for confirmation purposes and to unravel the biological link between genetic variants and the SCA manifestations. Gene expression studies are also of the utmost importance, in particular in the case of *VCAM1* and *ITGA4* variants to assess overall up- or down-regulation of the gene, as a consequence of the changes in the regulatory sequence. In *NOS3* functional analysis is also mandatory to evaluate enzymatic activity in different cellular environments. All studies undertaken to identify genetic modifiers of SCA sub-phenotypes are important to pinpoint essential pathways and mechanisms for SCA pathophysiology and to evaluate potential molecular targets to which direct innovative therapeutic strategies.

Acknowledgments

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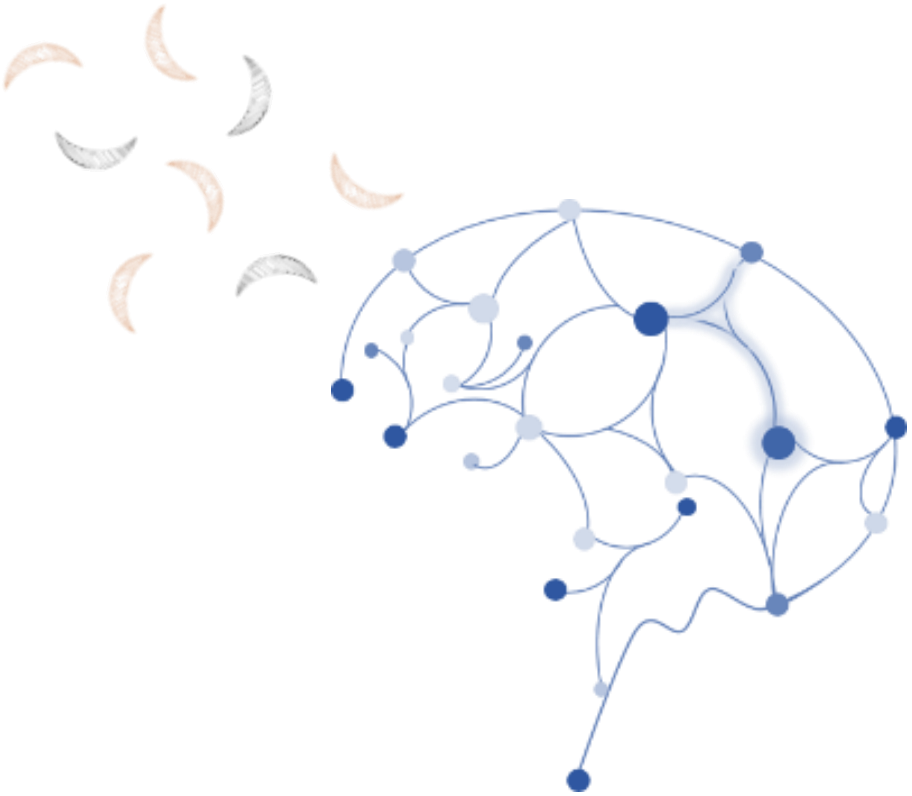


Functional Studies

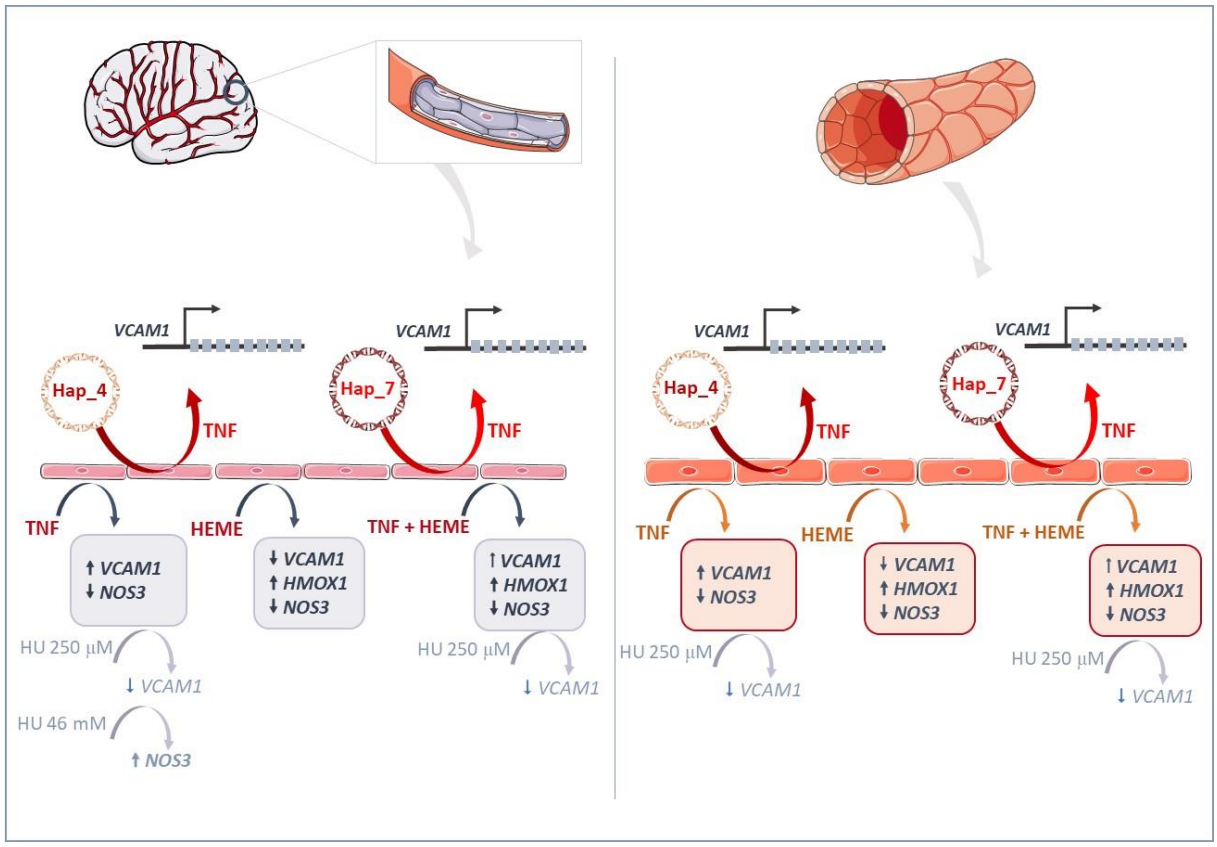
Part III

Chapter 4

DIFFERENTIAL ENDOTHELIAL CELL MODULATION BY *VCAM1*

Insights for Sickle Cell Anemia Vasculopathy

Graphical abstract



Based on:

Marisa Silva, Sofia Vargas, Andreia Coelho, and Paula Faustino. *Differential endothelial cell modulation by VCAM1: insights for sickle cell anemia vasculopathy*. Submitted to *Annals of Hematology*, April 2021.

4.1. SUMMARY

Endothelial dysfunction plays a major role in SCA's systemic vasculopathy, with upregulation of adhesion molecules (e.g., VCAM-1), decreased nitric oxide bioavailability, and oxidative stress. We aimed to assess the modulation role of proinflammatory and/or pro-oxidative stimuli on endothelial *VCAM1*, *NOS3*, and *HMOX1* expression. We also evaluated the effect of the main SCA therapeutic agent, hydroxyurea (HU), on that modulation.

Our results showed that two *VCAM1* promoter haplotypes, previously associated with pediatric cerebral vasculopathy and severe hemolysis in SCA, increased promoter activity in TNF-α-stimulated transfected EA.hy926 and HBEC, consistent with a higher *VCAM1* expression in macro and microvascular settings. In non-transfected cells, we also observed TNF-α-induced *VCAM1* overexpression as well as heme-induced overexpression of *HMOX1* in both cell models. Heme did not affect *VCAM1* nor *NOS3* expression and the latter was also not affected by TNF-α stimulus. Hydroxyurea treatment lowered TNF-induced *VCAM1* and *NOS3* expression but did not affect heme-induced *HMOX1* expression.

These data further indicate that *VCAM1* haplotypes we previously associated with pediatric cerebral vasculopathy and hemolysis in SCA, lead to higher VCAM1 expression affecting not only cerebral but also systemic vasculopathy risk. The differential endothelial expression of *VCAM1*, *NOS3*, and *HMOX1* also confirms their genetic modulation role in SCA systemic vasculopathy.

Keywords: vascular endothelium; vasculopathy; *VCAM1* promoter haplotypes; *TNF-α*; heme; hydroxyurea; sickle cell anemia.

4.2. BACKGROUND

As described earlier, multifactorial-like complex pathophysiology of SCA includes chronic hemolysis, inflammation and coagulation, vaso-occlusion, impaired microvascular blood flow, and ischemia/reperfusion injury that frequently leads to strokes and organ infarctions [151,158,225,403]. Vaso-occlusion – as a crucial initiator of ischemia/reperfusion injury damage effects [204] – and chronic hemolysis – as a sustained source of heme that acts as a pro-inflammation damage-associated molecular pattern molecule (DAMP) [404] – are thus fundamental pathophysiological processes.

The interaction through specific mediators between blood cells and the vascular endothelium and the subsequent endothelial response has been one of the most complex fields of research of SCA. Endothelial activation – loss of vascular integrity, expression of adhesion molecules (e.g., VCAM-1, selectins, ICAM-1), change to a pro-thrombotic state, and cytokine production – and endothelial dysfunction – impairment of endothelium-dependent vasodilation due to a reduction of the bioavailability of vasodilators, namely nitric oxide (NO), and/or an increase in endothelium-derived contracting factors – are key pathomechanisms of SCA. These disturbances of normal endothelial function and are triggered by pro-inflammatory stimuli, such as cytokines (e.g. TNF-α, IL-6, IL-4) and chemokines (e.g. CXCL-8), sickle RBCs, oxidative stress (including hypoxia), and infections [151,405].

Sickle RBCs are fragile, have lower deformability and high endothelial adhesion, which makes them prone to premature destruction. The subsequent (intravascular) hemolysis releases hemoglobin (Hb) and free heme with damaging effects in the vasculature [151,406,407]. Free Hb, as well as arginase-1, scavenge nitric oxide (NO) produced by the endothelial oxide synthase (eNOS or NOS3), lowering NO bioavailability [151]. Consequently, the vascular tone is disturbed and shifts towards

vasoconstriction [151,408]. Endothelial function and, ultimately, organ function are also impaired as a result. On the other hand, chronic inflammation in SCD arises, at least in part, from leukocytosis, with abnormally high numbers of leukocytes and monocytes secreting pro-inflammatory cytokines [409]. Free heme has been shown to stimulate cytokine (e.g., TNF-α, IL-6, IL-10), and adhesion molecules (e.g., ICAM-1) production, leukocyte activation, and migration, and oxidant production in sickle cell mice models [158,403,409]. These effects may be limited by the action of the heme-oxygenases, like the inducible heme-oxygenase-1 (HO-1), which degrades heme into carbon monoxide (CO), biliverdin, and iron (Fe^{2+})[403].

Endothelial activation and dysfunction have profound effects in organs like the brain, where stroke is the most severe end of the cerebral vasculopathy spectrum. The endothelial upregulation of adhesion molecules, such as vascular adhesion molecule-1 (VCAM-1), as well as impaired NO bioavailability and a pro-oxidant milieu, affect both the macro and microvasculature. The highly complex and heterogeneous nature and physiology of the vascular endothelium also exert an important impact on the pleiotropic-like phenotype. Treatment is complex and despite hydroxyurea (HU) is the most established FDA-approved therapeutic option, its full action spectrum is still unknown.

In previous studies, we identified specific *VCAM1* promoter haplotypes associated with stroke and stroke risk, as well as with hemolysis in SCA children of Sub-Saharan ancestry [354,382]. Our goals in the present work were to perform functional studies, to assess the modulator effects of those haplotypes on *VCAM1* promoter activity in macro and microvascular endothelial cell response to cytokine (TNF-α) stimulus. We also aimed to evaluate the modulation effects of the same pro-inflammatory stimulus alone or in combination with a pro-oxidative stimulus (hemin, or heme) on the *VCAM1* gene

expression. Two other genes with predominantly endothelial expression and also involved in SCA pathophysiology – *HMOX1* (inducible) and *NOS3* (constitutive) – were included for comparison purposes. Finally, HU treatment was applied in different dosages to assess its potential in counteracting the damaging effects on the expression of the three genes and ultimately its gene expression modulator role on macro- and microvascular endothelial cells.

4.3. METHODOLOGY

4.3.1. Plasmid Constructs

VCAM1 promoter activity was assessed using reporter constructs that included previously described promoter haplotypes with a significant association with pediatric stroke [382] and hemolysis in SCA [354]. Three haplotypes were chosen (Hap_1, Hap_4, and Hap_7, Table 4.1). The *VCAM1* promoter sequence we considered spanned from an upstream regulatory region 2155 bp sequence to the start site. The fragment was amplified from genomic DNA, obtained from patients with SCA, using PCR, with the forward primer 5'-TTGACTCGAGCTGATCATTCATTAATTCTGC-3', containing the *Xho* I linker, and the reverse primer 5'-TAATAGATCTGTCGTGATGAGAAAATAGTG-3' containing the *Bgl* II linker. The promoter sequence was cloned into the pGL4.luc10 promoterless vector (Promega). Digestion of both the pGL4.luc10 empty vector and the PCR products with the *Xho* I and *Bgl* II endonucleases allowed to obtain the vector+promoter_haplotype constructs. Ligation of the PCR products into the pGL4.luc10 vector was performed using the Rapid DNA Ligation Kit (Roche, Manheim, Germany). Sanger sequencing prior to the transfection experiments confirmed the constructs' sequences.

Table 4.1. *VCAM1* promoter haplotypes used in this study

***VCAM1* Variant**	***VCAM1* Haplotypes**						
	Hap_1	**Hap_2**	**Hap_3**	**Hap_4**	**Hap_5**	**Hap_6**	**Hap_7**
rs1409419 (T>C)	C	C	C	C	C	C	T
rs3917024 (C>T	C	C	C	C	C	T	C
rs3917025 (CT>del CT)	CT	CT	CT	CT	del CT	del 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

4.3.2. Cell Cultures

EA.hy926 and human brain endothelial (HBEC) cell lines (ATCC CRL-2922 and ATCC CRL-3245, respectively) were acquired from the American Type Culture Collection (Manassas, VA, USA). EAhy926 cells were maintained in DMEM Glutamax™ Hi-Glucose medium (Invitrogen, Carlsbad, CA, USA), supplemented with 10% (v/V) heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA). HBEC were cultured in flasks previously coated with a 0.1% gelatin solution (ATCC, Manassas, VA, USA), with DMEM Hi-Glucose medium DMEM Glutamax™ Hi-Glucose medium (Invitrogen, Carlsbad, CA, USA), supplemented with 40 μg/mL endothelial cell growth supplement (Corning®, Bedford, MA, USA) and 10% (v/v) FBS. Cell lines were used for all assays in passages 2 to 6, *Mycoplasma*-tested, and characterized by STR profiling and karyotyping (Supplementary Material). Cell viability was evaluated by Trypan blue exclusion, before passage to 35 mm 6-well plates.

4.3.3. Transient Transfections of *VCAM1* Haplotype Constructs

Transient transfections were performed in 90% confluent EA.hy926 and HBEC cells, on 35 mm 6-well plates using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions, and using 1 μg of the test construct. Cell viability was evaluated by Trypan blue exclusion. phRL-TK vector (Promega, Maddison, WI, USA) was co-transfected to use as an internal control of transfection efficiency. Four hours after transfection, the medium was replaced with fresh medium, and sixteen hours later cells were treated with 20ng/mL TNF-α (Recombinant Human TNF-α Lyophilized, Gibco, Life Technologies, Carlsbad, CA, USA). Cells were harvested after 8h for luciferase reporter assays and RNA extraction.

4.3.4. Dual-Luciferase Reporter Gene Assays

VCAM1 promoter activity was measured using luciferase activity in the above described EA.hy926 macrovascular and HBEC microvascular endothelial cells. Luciferase activity was measured using the Dual-Glo® Luciferase Assay System (Promega, Maddison, WI, USA) in a Glomax® 96-well microplate luminometer (Promega, Maddison, WI, USA).

Firefly luciferase (FLuc) activity was normalized against that of *Renilla* luciferase (RLuc) activity. Promoter activities were estimated relative to that of the pGL4.luc10 vector without the promoter sequence ("empty vector"). The luciferase activity for each reporter construct was assayed in triplicate, in at least three independent experiments. The luciferase values are presented as fold change after normalization to phRL-TK values, using "empty" pGL4-transfected EC luminescence values as controls. Fold-change was calculated using the expression ΔFold Activity = Average (Fluc/Rluc) from sample B / Average (Fluc/Rluc) from sample A.

4.3.5. RNA Isolation and Gene Expression Assays

Cells were grown until 90% confluence and then exposed to TNF-α (20 ng/mL) [410] stimulation for 8h. Hydroxyurea (HU) treatment (250 μM and 46 mM) was applied to cells, with or without previous TNF-α stimulation, for 4h. HU 250 μM concentration was based on similar studies in endothelial cells [410–412], and the use of 46 mM concentration was based on the maximum tolerated dose of 35 mg/kg/day recommended for pediatric patients [413], adjusted to human body density. Hemin (70 μM) treatment [317,414] was applied in combination or not with HU for 4h. Total RNA was extracted from cultured cells using the NucleoSpin® II RNA kit, as recommended by the manufacturer (Macherey-Nagel, Düren, Germany). First-strand cDNA synthesis from 2 μg of total RNA was carried out using random hexamers and the SuperScript® First-Strand Synthesis System, following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The synthesized cDNA samples were kept at -20°C until the real-time quantitative PCR (RT-qPCR) experiments were performed.

RT-qPCR was performed in a 7500 Real-Time PCR System using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). Primers were designed for *VCAM1*, *NOS3*, and *HMOX1* genes, as well as for the housekeeping gene *GAPDH* (Appendix Table A.1). Gene expression was measured by relative quantification, as follows: after determination of the cycle threshold (Ct), fold change was obtained calculating the $2^{\Delta\Delta Ct}$ value, where $\Delta Ct = Ct_{reference} - Ct_{target}$, and $\Delta\Delta Ct = \Delta Ct_{treated} - \Delta Ct_{control}$. The *GAPDH* was the normalizer/reference gene. Each sample was quantified in triplicate, in at least two different experiments.

4.3.6. Statistical Analysis

Data are expressed as means ± SEM. Two groups were considered significantly different if the *p*-value was <0.05. Multiple group comparisons were performed using one-way ANOVA and post-hoc Tukey's or Dunnett T3 tests (*p*-value ≤0.05 for significant differences). Statistical analysis was conducted using SPSS software version v.25.0 (IBM Inc., Chicago, IL, USA).

4.4. RESULTS

4.4.1. *VCAM1* Haplotypes Show Differential Promoter Activities in TNF-α Stimulated Endothelial Cells

To assess if the previously identified *VCAM1* promoter haplotypes (Hap_1, Hap_4, and Hap_7, Table 4.1) led to differences in promoter activity on endothelial cells, we transfected the corresponding pGL4.luc10-*VCAM1*_promoter constructs into two EC models (EA.hy926 and HBEC). Hap_1, with no previous significant association with cerebral vasculopathy or hemolysis, was used for comparison purposes. EA.hy926, a somatic cell hybrid cell line engineered from human umbilical vein endothelial cells, has been frequently used in EC studies and was chosen to simulate macrovascular EC response. Since one of our aims was also to investigate potential cerebral vasculopathy effects, we used human brain endothelial cells (HBEC) as a cerebral microvascular endothelial model. TNF-α was applied as the cytokine pro-inflammatory stimulus to activate *VCAM1* expression in both models.

Luciferase activity revealed that the three haplotypes analyzed show differential effects on promoter activity. EA.hy926s and HBECs transfected with Hap_4 and Hap_7, and stimulated with TNF-α, showed increased luciferase activity when compared with cells transfected with the "empty" vector (Fig. 4.1). Hap_4, previously associated with

severe hemolysis in SCA pediatric patients, led to a 1.71±0.06-fold increase in EA.hy926, while in HBECs the increase was slightly (though not significantly) lower (1.29±0.50). The induction in luciferase activity was even more expressive as a result of Hap_7 transfection in a pro-inflammatory milieu (1.77±0.49-fold in EA.hy926 and 2.85±0.73-fold in HBEC, respectively). This haplotype, previously found by our group to be associated with stroke and pediatric cerebral vasculopathy risk in SCA, led to significantly higher promoter activity on HBECs ($p<0.001$). Although both haplotypes affected the two types of EC tested, the impact of Hap_7 in the cerebral microvascular model was twice as stronger as the one from Hap_4 ($p<0.001$). Conversely, and despite a slight increase on EA.hy926 cells (1.17±0.12-fold change) and a decrease on HBECs (0.89±0.16-fold change), Hap_1 did not lead to significant effects in *VCAM1* promoter activity.

4.4.2. TNF-α and Hemin Show Opposite Effects on *VCAM1* Activation

Besides assessing the effects of cytokine stimulation on *VCAM1* expression, we also applied hemin (ferric heme) to simulate the hemolytic vascular environment, where free heme acts both as a pro-inflammatory and as an oxidant stimulus.

We did not observe *VCAM1* expression in non-stimulated EA.hy926 cells and therefore TNF-α stimulated cells were used as controls for the relative quantification (Figs. 4.2 A and B). All treatments that did not include the cytokine had the same effect on this cell line. Combination of TNF-α with hemin and/or HU resulted in the down-regulation of *VCAM1* expression. The most striking result was associated with TNF-α/hemin combined stimulation. The oxidant stimulus led to a 0.12-fold change in *VCAM1* mRNA

expression ($p \leq 0.05$). The addition of HU significantly enhanced this effect, namely the 250 μM dosage ($p < 0.01$).

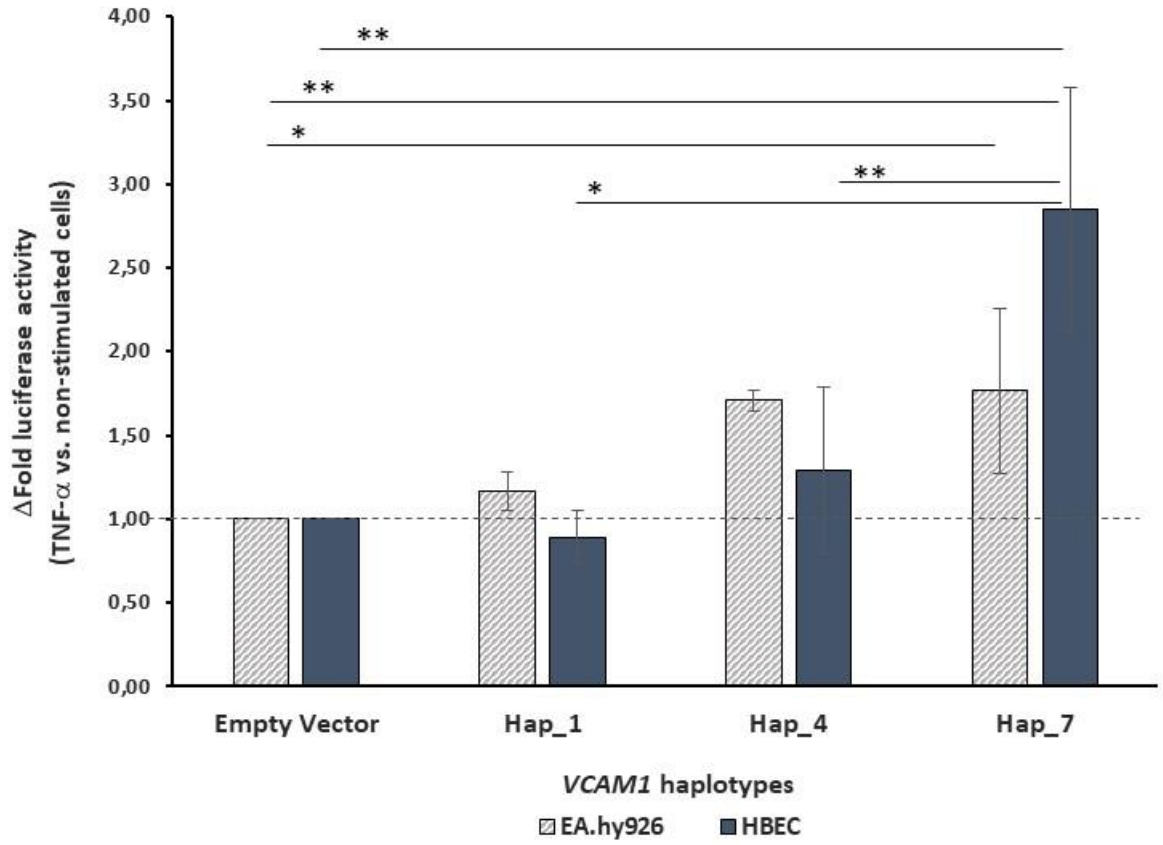


Figure 4.1. ***Estimates of relative luciferase activity (ΔFold activity) on EA.hy926 and HBEC cell lines transfected with*** **VCAM1** ***promoter haplotypes Hap_1, Hap_4, and Hap_7, respectively.*** Fold-changes of *VCAM1* promoter activity were calculated using the following equation: ΔFold activity = [Average (Fluc/Rluc) from sample A] / [Average (Fluc/Rluc) from the sample "empty vector"]. Data are expressed as mean±SEM of at least two replicates, performed in at least three independent experiments. Statistical significance: *$p \leq 0.05$; **$p < 0.01$.

On the other hand, the HBECs response was markedly different (Figs. 4.2 C and D). We observed *VCAM1* basal expression in non-stimulated cells although at lower relative levels when compared with the TNF-α control ($p < 0.001$). Hemin also induced

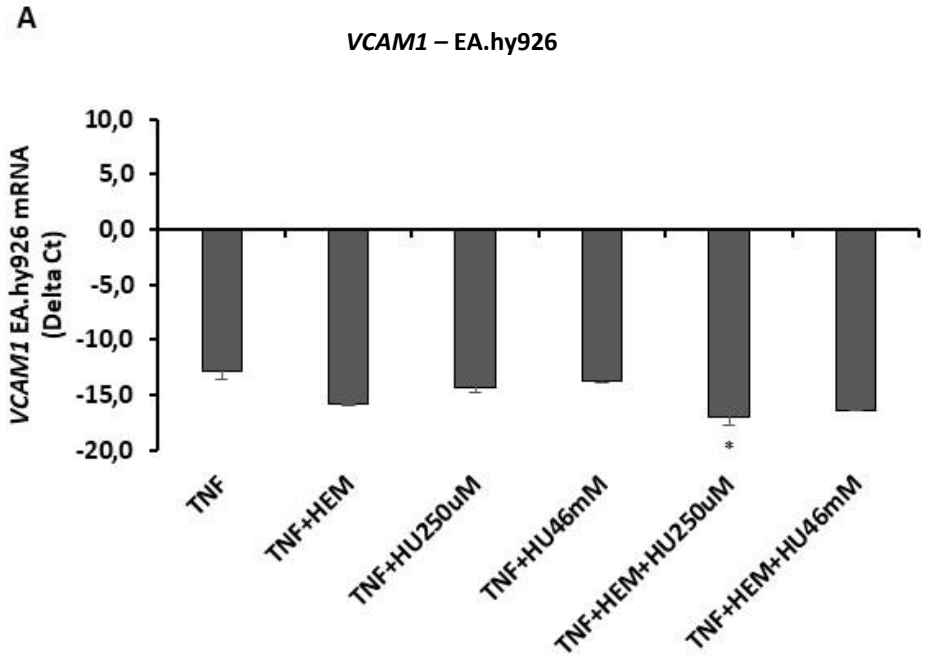
expression in these cells but at even lower relative levels ($p<0.001$). Contrary to what we observed on EA.hy926 cells, the combined exposure to TNF-α and hemin led to a 1.4-fold change in *VCAM1* expression in HBECs, although this upregulation was not statistically significant ($p>0.05$). HU treatment did not counteract this effect, and the 46mM concentration heightened it even further ($p<0.001$). Conversely, this treatment downregulated expression on hemin-stimulated cells, whether in the presence or absence of TNF-α ($p<0.001$). Treatment with HU alone was not associated with detectable relative levels of *VCAM1* mRNA.

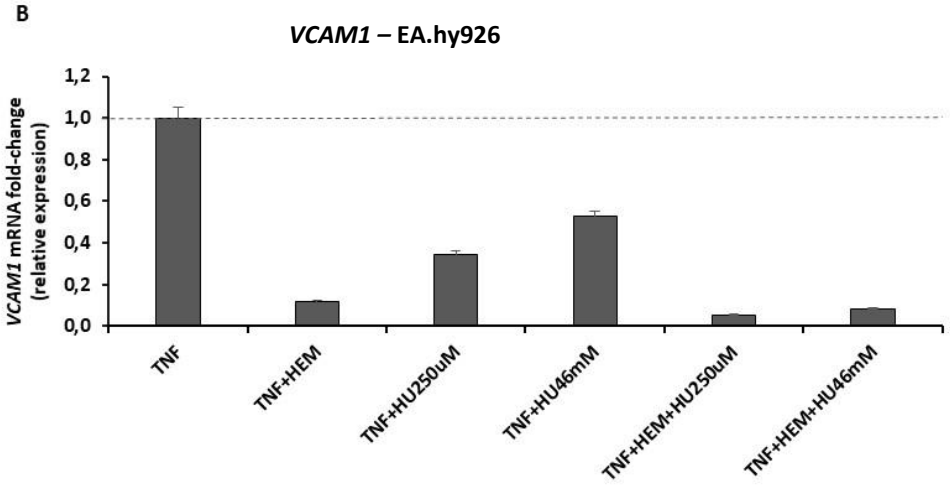
4.4.3. Hemin Treatment Alone, or in Combination, Markedly Induces *HMOX1* but not *VCAM1* Gene Expression

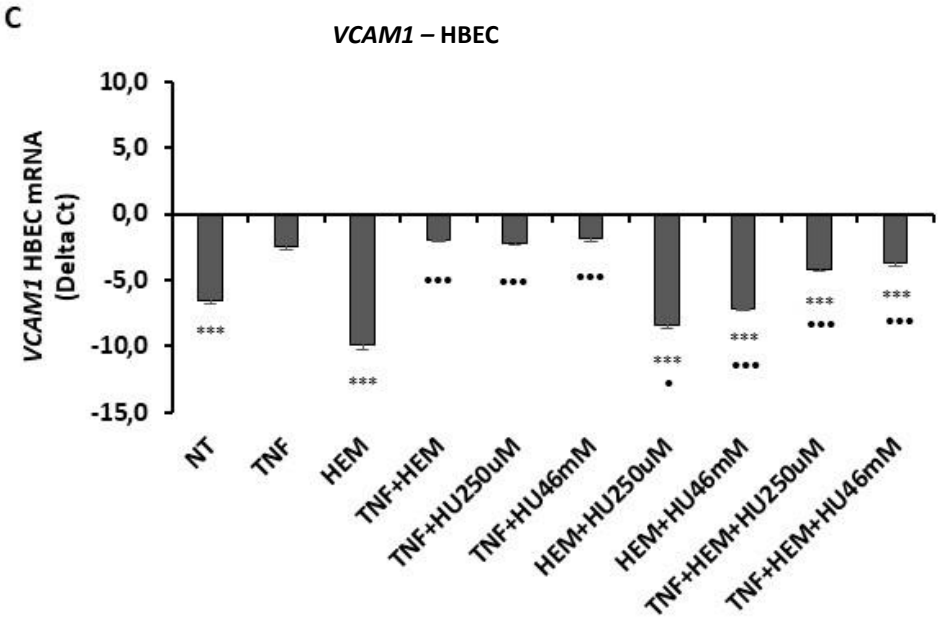
Hemin, in the presence or absence of TNF-α, was shown to markedly induce *HMOX1* expression, in both cell models (Figs. 4.2 E-H). In EA.hy926 vs. controls, there was a 255.1-fold change in expression ($p<0.001$), due to hemin alone. In hemin-stimulated HBECs, the *HMOX1* mRNA levels upregulation was even more pronounced, with a 277.2-fold change ($p<0.001$) when compared to non-stimulated cells. This effect was not significantly altered when treatment with either of the two different HU concentrations (250 μM or 46 mM) was applied. Moreover, HU alone did not significantly activate the response of this antioxidant gene when compared with the hemin effect in EA.hy926 ($p>0.05$) and HBECs ($p>0.05$). We even observed a reduced expression resulting from HU in combination with TNF-α and/or hemin ($p<0.05$ and $p<0.001$, respectively).

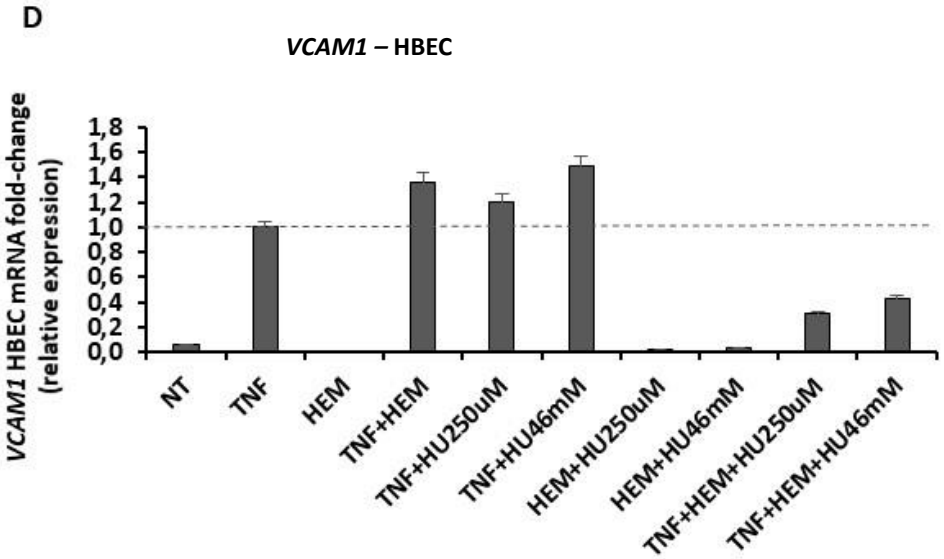
4.4.4. HU has a Dosage-Dependent Effect on *NOS3* Expression

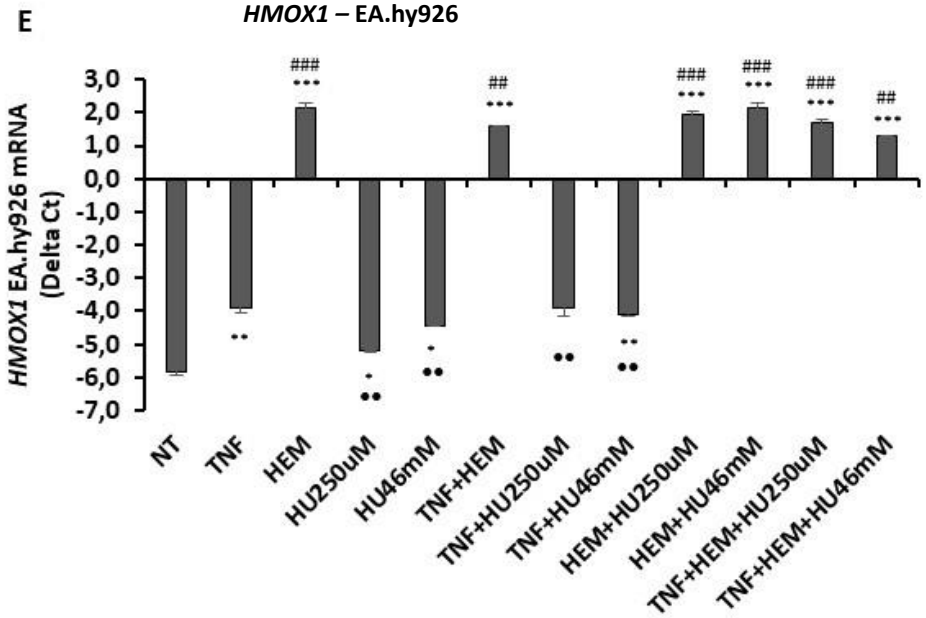
NOS3 mRNA levels were found to be down-regulated in the majority of conditions tested (Figs. 4.2 I-L). However, differential dosage-dependent *NOS3* expression was observed in response to HU. This effect was more noticeable in cerebral microvascular cells. HBECs treated with 46 mM HU showed a significant increase in *NOS3* expression ($p \leq 0.05$), while the 250 μM HU treatment led to a non-significant decrease. The dosage-dependent differential modulation was also observed in cells after stimulation with TNF-α alone, although to a lesser extent. Nevertheless, HU treatment was not sufficient to revert decreased *NOS3* expression resulting from exposure to combined TNF-α/hemin stimulus.




B
VCAM1 – EA.hy926
VCAM1 mRNA fold-change (relative expression)
1,2
1,0
0,8
0,6
0,4
0,2
0,0
TNF
TNF+HEM
TNF+HU250uM
TNF+HU46mM
TNF+HEM+HU250uM
TNF+HEM+HU46mM

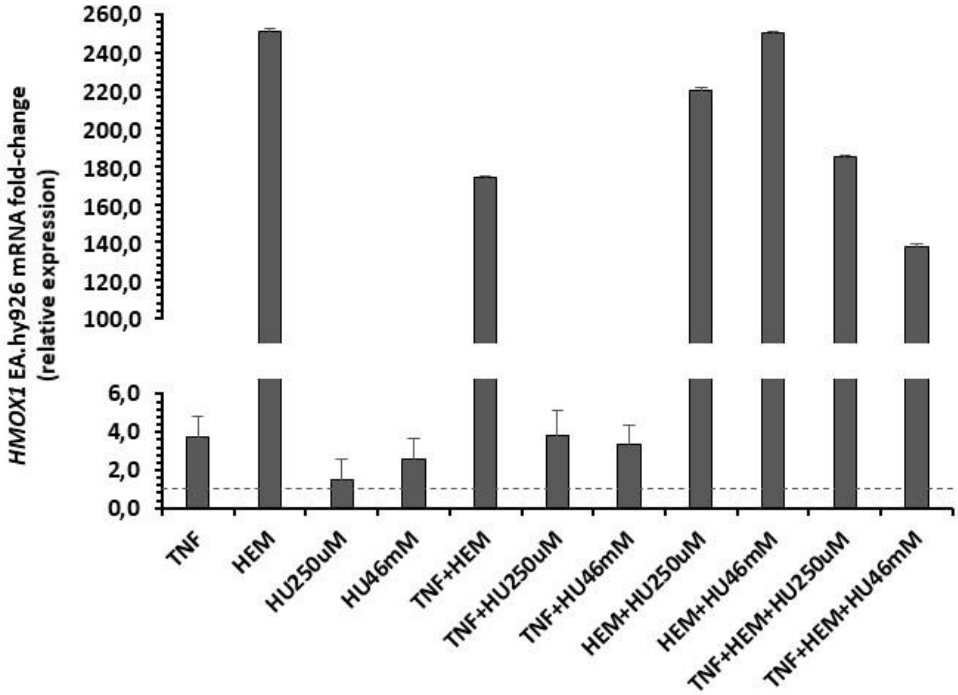

C
VCAM1 – HBEC
VCAM1 HBEC mRNA (Delta Ct)
10,0
5,0
0,0
-5,0
-10,0
-15,0
NT
TNF
HEM
TNF+HEM
TNF+HU250uM
TNF+HU46mM
HEM+HU250uM
HEM+HU46mM
TNF+HEM+HU250uM
TNF+HEM+HU46mM


D
VCAM1 – HBEC
VCAM1 HBEC mRNA fold-change (relative expression)
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
NT
TNF
HEM
TNF+HEM
TNF+HU250uM
TNF+HU46mM
HEM+HU250uM
HEM+HU46mM
TNF+HEM+HU250uM
TNF+HEM+HU46mM


E
HMOX1 – EA.hy926
HMOX1 EA.hy926 mRNA (Delta Ct)
3,0
2,0
1,0
0,0
-1,0
-2,0
-3,0
-4,0
-5,0
-6,0
-7,0
NT
TNF
HEM
HU250uM
HU46mM
TNF+HEM
TNF+HU250uM
TNF+HU46mM
HEM+HU250uM
HEM+HU46mM
TNF+HEM+HU250uM
TNF+HEM+HU46mM

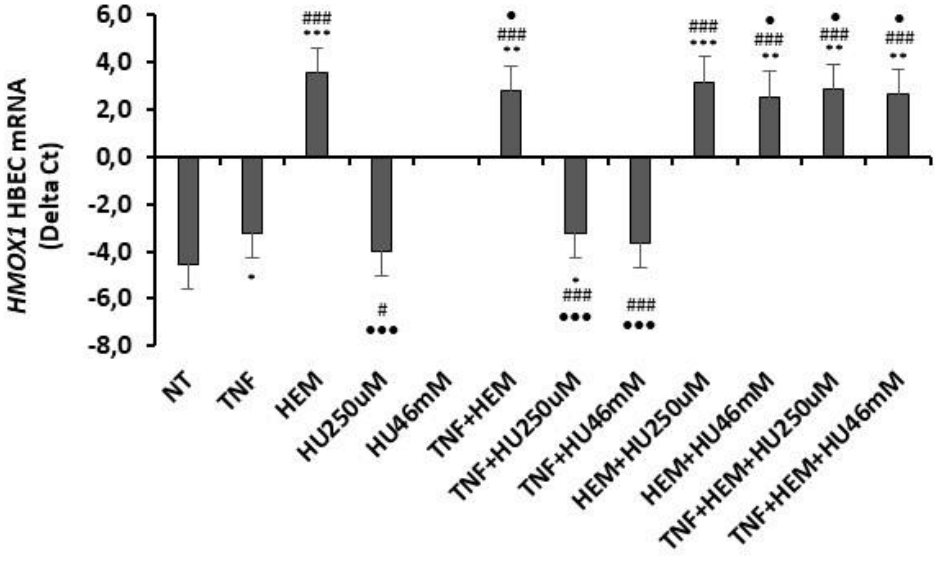
F

***HMOX1* – EA.hy926**



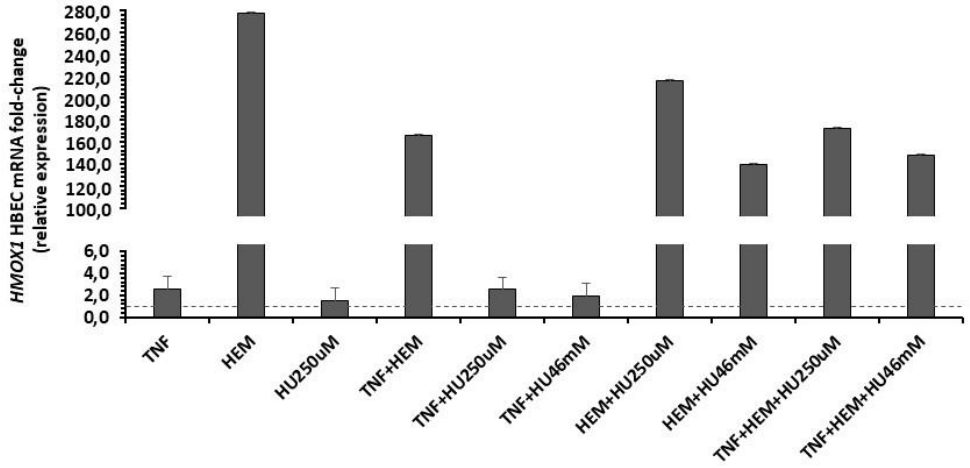
G

***HMOX1* – HBEC**



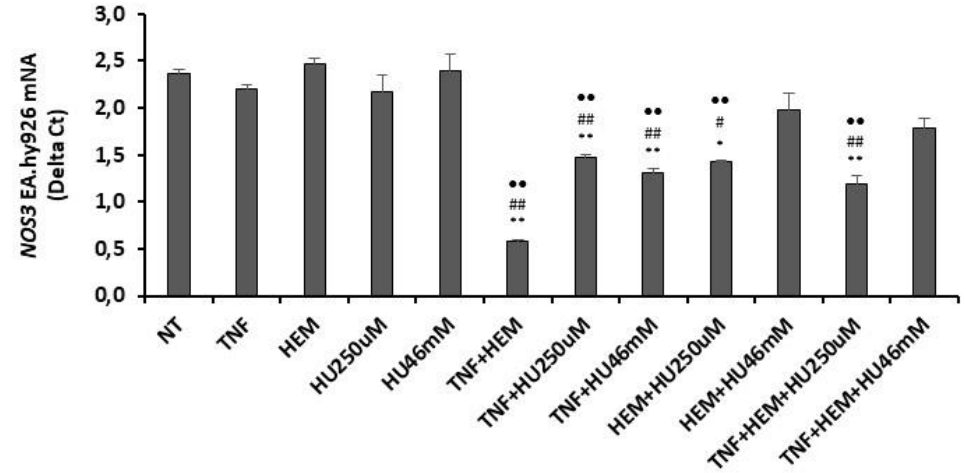
H

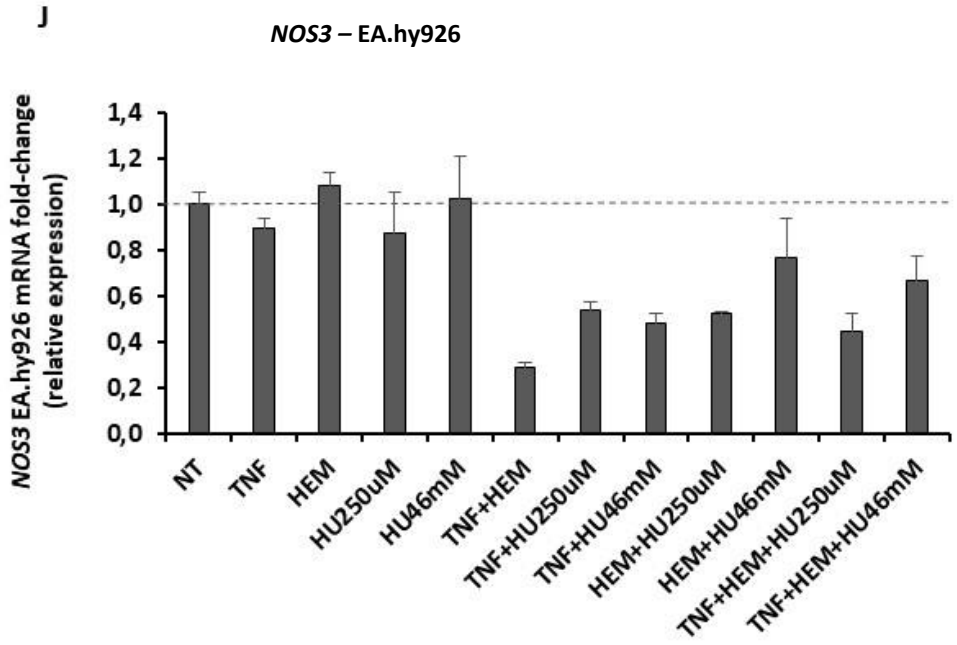
***HMOX1* – HBEC**

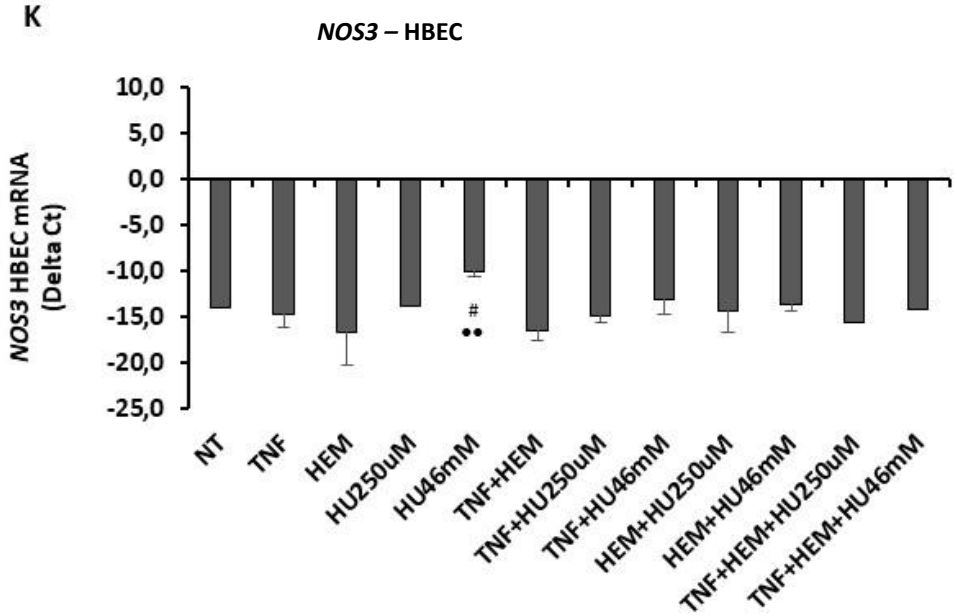


I

***NOS3* – EA.hy926**




J
NOS3 – EA.hy926
NOS3 EA.hy926 mRNA fold-change (relative expression)
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
NT
TNF
HEM
HU250uM
HU46mM
TNF+HEM
TNF+HU250uM
TNF+HU46mM
HEM+HU250uM
HEM+HU46mM
TNF+HEM+HU250uM
TNF+HEM+HU46mM


K
NOS3 – HBEC
NOS3 HBEC mRNA (Delta Ct)
10,0
5,0
0,0
-5,0
-10,0
-15,0
-20,0
-25,0
#
••
NT
TNF
HEM
HU250uM
HU46mM
TNF+HEM
TNF+HU250uM
TNF+HU46mM
HEM+HU250uM
HEM+HU46mM
TNF+HEM+HU250uM
TNF+HEM+HU46mM

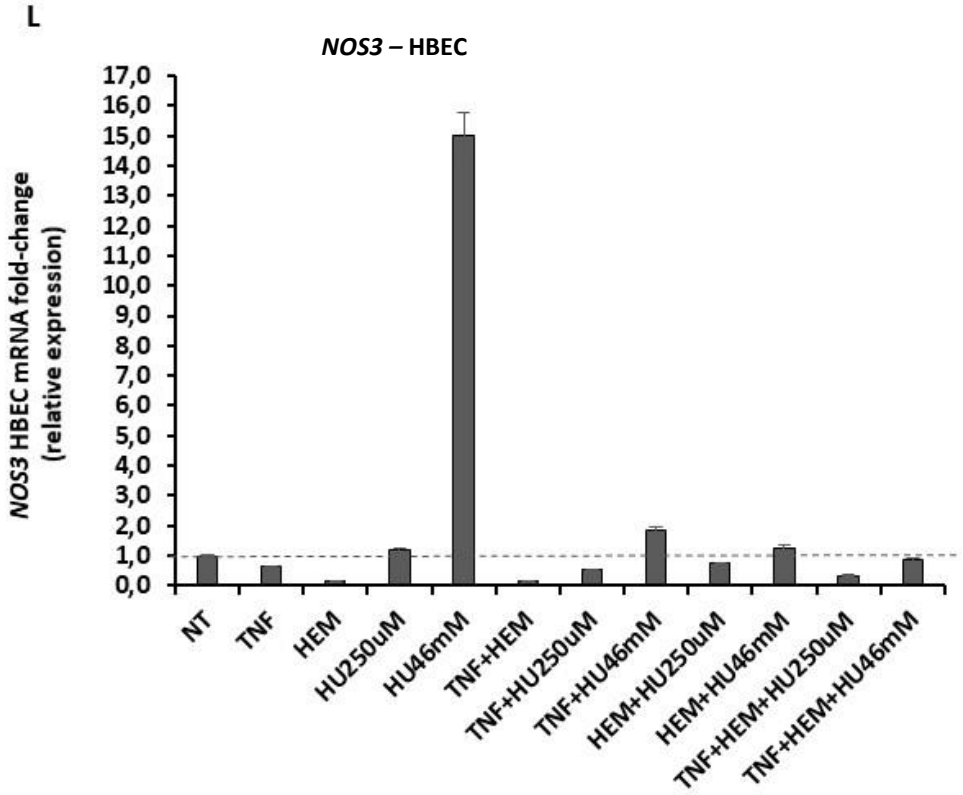


Figure 4.2. EA.hy926 and HBEC expression of *VCAM1*, *HMOX1*, and *NOS3*, after TNF-α (20 ng/mL) and/or hemin (70 μM) stimulation, in the presence or absence of hydroxyurea treatment (250 μM or 46mM) for 4h. A) *VCAM1* EA.hy926 mRNA ΔCt; B) *VCAM1* EA.hy926 mRNA relative expression (fold-change); C) *VCAM1* HBEC mRNA ΔCt; D) *VCAM1* HBEC mRNA relative expression (fold-change); E) *HMOX1* EA.hy926 mRNA ΔCt; F) *HMOX1* EA.hy926 mRNA relative expression (fold-change); G) *HMOX1* HBEC mRNA ΔCt; H) *HMOX1* HBEC mRNA relative expression (fold-change); I) *NOS3* EA.hy926 mRNA ΔCt; J) *NOS3* EA.hy926 mRNA relative expression (fold-change); K) *NOS3* HBEC mRNA ΔCt; and L) *NOS3* HBEC mRNA relative expression (fold-change). Results are expressed as mean±SEM of at least three replicates. Gene expression values were calculated by relative quantification as follows: fold-change = $2^{\Delta\Delta Ct}$, where $\Delta Ct = Ct_{reference}-Ct_{target}$, and $\Delta\Delta Ct = \Delta Ct_{treated}-\Delta Ct_{control}$. Controls: TNF-α stimulated cells for *VCAM1*; and non-treated cells for *NOS3* and *HMOX1*. Data were normalized to represent fold-change when compared to control for each gene (TNF for *VCAM1* and NT for *HMOX1* and *NOS3*). Statistical significance measured by one-way ANOVA followed by Dunnett T3 or Tukey post-hoc test: treatment vs. control *p<0.05, **p<0.01, ***p<0.001; treatment vs. TNF alone $^{\#}$ p<0.05, $^{\#\#}$ p<0.01, $^{\#\#\#}$ p<0.001; treatment vs. hemin alone •p<0.05, ••p<0.01, ••• p<0.001. Dashed lines represent the baseline fold-change corresponding to the control. NT: non-treated; TNF: TNF-α 20 ng/mL; HEM: hemin 70 μM; HU250uM: hydroxyurea 250 μM; HU46 mM: hydroxyurea 46 mM.

4.5. DISCUSSION

The present study aimed to assess modulation of *VCAM1* gene expression, on macrovascular as well as brain microvascular ECs, under pro-inflammatory and pro-oxidative stimuli. This was performed in EC transfected or not with pGL4.luc10 constructs containing specific *VCAM1* promoter haplotypes, after TNF-α stimulus combined or not with hemin exposure. These *VCAM1* promoter haplotypes were selected to be further investigated due to their previously observed pathogenic association with cerebral vasculopathy, stroke risk, and severe hemolysis in SCA [354,382]. Furthermore, we evaluated if and how that modulation changed under HU treatment, the main therapeutic agent used in SCA. For comparison purposes, our assessment included *NOS3* and *HMOX1* gene expression under the same stimuli and HU treatment.

In these functional studies, we observed differential promoter activity associated with the presence of two of those haplotypes. Specifically, our study has shown that the presence of Hap_ 7 in TNF-α-stimulated ECs led to markedly higher promoter activity. This will potentially result in an increased *VCAM1* upregulation, in TNF-α-stimulated ECs. The impact on cerebral microvascular ECs is even more noteworthy due to its potential implications for cerebral vasculopathy in SCA and is in line with our findings in previous association studies [382]. Furthermore, the differential response to both Hap_4 and Hap_7 observed in macro- as well as cerebral microvascular endothelial cell also suggests that these genetic modulators may be critical for the (systemic) vasculopathy phenotype typical in SCA, and not only for the cerebral vasculopathy. *VCAM1* activation is mainly under transcriptional control. Original studies of the 2,300bp *VCAM1* promoter region in ECs identified two NF-κb binding sites whose activation is TNF-α and interleukin-1 (IL-1) dependent [107,124]. This activation was

found to be EC-specific. Our previous transcription factor binding site *in silico* analyses indicated that neither of the variant alleles composing Hap_4 and Hap_7 would affect the NF-κB sites [382,415]. The potential gain of EVI-1, BarX2, or Oct1 transcription factor binding sites indicated by those studies does not directly explain the increase in *VCAM1* expression we observed, as these transcription factors individually usually work as gene expression repressors [416–418]. However, we cannot exclude a potential interaction/cooperation between one of them and the TNF-α/NF-κB axis.

TNF-α causes degradation of the glycocalyx [419], leading to several damaging effects including increased production of adhesion molecules to promote red blood cell adhesion [420]. The expression of VCAM-1 is usually negligible in resting ECs but it rapidly increases after TNF-α exposure, which was corroborated by the differences we observed between non-treated and treated ECs. TNF-α also leads to reduced NO bioavailability. This occurs as a result of the activation of endothelial arginase (which depletes eNOS from its substrate arginine) [421] and endothelial NADPH oxidase (lowering BH4 and leading eNOS to generate superoxide) [422]. We found down-regulation of *NOS3* expression in TNF-α-stimulated EA.hy926 cells with the majority of treatments applied. This effect was counteracted by 46 mM (but not 250 μM) HU treatment. TNF-α modulation was found to affect the *NOS3* gene promoter by downregulating *NOS3* expression in ECs [423]. Moreover, this modulation has been shown to occur at the post-transcriptional level, with mRNA destabilization through binding of the eEF1A protein to the 3'UTR sequence of *NOS3* mRNA [424]. We also observed the TNF-α *NOS3* downregulation effect in HBECs. In this case, the reduction was even more significant and only counteracted by treatment with 46 mM HU. Besides comprising the brain vasculature, cerebral ECs actively participate in central nervous

system (CNS) inflammation. ECs of capillaries and post-capillary venules in the CNS are close to astrocytes, pericytes, neuronal cells, and perivascular microglia (reviewed in [425]). They express TNFR1 and TNFR2, the receptors that bind TNF-α, thus modulating endothelial tight-junctions and cytoskeleton rearrangement. Moreover, they have been shown to regulate iNOS and ICAM-1 production by an autocrine TNF-α-dependent pathway, as a response to infection [426]. The ability to produce iNOS in response to external stimuli, hence providing an alternative source of NO, may explain the marked downregulation of *NOS3* expression.

Hemin (ferric heme) has pro-oxidant and pro-inflammatory effects and has been used to simulate a sudden increase in intravascular hemolysis and blood flow stasis in post-capillary veins in sickle mice [158,403]. Heme was shown to activate Toll-like receptor-4 (TLR-4) signaling leading to delivery of P-selectin and vWF to the vessel wall and NF-κB activation, with both of these events leading to vaso-occlusion in sickle, but not in normal, mice [158]. The authors also demonstrated that antibody-blocking of VCAM-1, α4β1integrin, P-selectin, E-selectin, vWF, ICAM-1, PECAM-1, and αVβ3 integrin inhibited heme-induced stasis. Furthermore, they also found that the presence of HbS was crucial to the manifestation of the heme-induced damaging effects. On the other hand, Belcher and colleagues observed VCAM-1 decreased expression and NF-κB activation in sickle lungs, liver, and spleen, after a 3-day treatment with hemin injections in sickle cell mice [427]. They concluded that the results were consistent with the inflammation and adhesion inhibition provided by the hemin-induced upregulation of HO-1 expression. In our study, we did not observe an increase of *VCAM1* expression in hemin-treated EA.hy926 ECs, and in HBEC the effect was lower than the one induced by TNF-α. These outcomes are in line, not only with the hemin-induced *HMOX1* upregulation but also with the absence of HbS, to which ECs are usually

exposed in SCD patients. In addition, the variation observed in *VCAM1* expression between EA.hy926 and HBEC may reflect local factors specific to the nature of the two cell types. Heterogeneity is a well-known characteristic of ECs, as they can differ in terms of intercellular tight junctions, morphology, mediator release, antigen presentation, and /or stress response [58]. HO-1 production has been shown to be upregulated by hemin in monocytes, where it has a protective antioxidant effect by inhibiting apoptosis [427,428]. As stated above, our findings are consistent with those observations, with a marked increase in *HMOX1* expression after hemin treatment. Both EC models show similar upregulation, thus suggesting that there is little difference in the levels by which this antioxidant system operates in the macrovascular and brain microvascular environments. Conversely, we observed a marked *NOS3* downregulation in HBECs as a result of heme exposure. This may also occur as a result of the alternative iNOS activation described above.

HU modulation on gene expression was assessed by treating ECs with two different HU concentrations – 250μM and 46mM. The 250μM HU treatment has been applied in similar studies that have assessed gene expression on endothelial cells [410–412] and based on plasma concentrations observed in patients treated with HU therapeutic dosages [429]. The HU 46mM concentration was based on the maximum tolerated dose of 35 mg/kg/day recommended for pediatric patients [413], adjusted to human body density. Moreover, we combined HU treatment with hemin at 70 μM, to assess EC response in pro-oxidative conditions, based on recent reports [317,414]. The modulating effect of HU on *VCAM1* expression, in both EC types, was limited by the pro-inflammatory and pro-oxidant endothelial context. In neither of the two dosages did the drug reduce *VCAM1* mRNA relative levels to baseline values. The impact was even lower when compared with levels after hemin stimulus. Traditionally, circumventing the

increased endothelial expression of this adhesion molecule has been performed with the use of drugs targeting its blood cell counter-ligand VLA-4 [130] or by antibody blocking of *VCAM1*. Blockade of TNF-α itself has also been proposed [190].

For *NOS3*, differential HU modulation was dose-dependent, consistent with what has been described by da Guarda and colleagues [411]. Only the concentration equivalent to the maximum recommended dose in the pediatric SCA setting (46 mM) was associated with increased expression, namely in microvascular brain ECs. This suggests that the HU in NO bioavailability, crucial to ameliorate the negative impact of hemolysis in SCA, does not result from direct *NOS3* upregulation. The HU effect in increasing eNOS levels is more likely to be post-translational, as the proteasomal protection from degradation mechanism proposed by other authors [310,411]. Furthermore, as described in chapter 2, we have previously identified *NOS3* variants associated with a protective effect on pediatric cerebral vasculopathy in SCA. Specifically, intron 4_27bp VNTR allele 4b (5 repeats), haplotype V (rs2070744_T / VNTR_4b / rs1799983_G) were associated to silent cerebral infarct protection, while haplotype VII (rs2070744_T / VNTR_4c / rs1799983_T) showed a putative protective effect on cerebral vasculopathy as a whole. The impact of HU we now observed on *NOS3* expression may further potentiate this protective effect, not directly but in an additive modulation model. Further studies on SCA patients' samples are warranted to further define the specific mechanism.

Additionally, and in line with what has recently been described for PBMCs and HUVECs [317], we found no effect of HU on *HMOX1* expression, either in EA.hy926 cells or HBECs. This reiterates the hypothesis that the activation of *HMOX1* in micro and macrovascular ECs follows an alternative mechanism of antioxidant response not susceptible to HU action.

Limitations to our study include the possibility of post-translational modification, (already known to occur for *NOS3*, for example), which may further affect protein levels. Additionally, the fact that endothelial cells are heterogeneous and are influenced by surrounding tissues also has to be considered before extrapolating to specific organ-damage mechanisms. Despite these limitations, our observations further reiterate the differential modulation of endothelial cell expression at the level of adhesion molecule production, nitric oxide production, and cytoprotective antioxidant systems. Moreover, and despite the overall benefits of HU in several SCA manifestations, there are still pathophysiological mechanisms that are not susceptible to its disease-modifying effects. This assumes particular importance for devising therapies for patients non-responsive to HU. Further knowledge of the mechanisms by which the differential modulation of these genes occurs is crucial. The impact on SCA is of particular importance for the more recent multiagent therapeutic approaches.

Acknowledgments

The authors wish to thank: Patrícia Barros and Vânia Gonçalves for support in the molecular cloning protocols; the colleagues of the Innovation and Technology Unit, for the *VCAM1*-promoter-constructs' sequencing analyses; José Ferrão for support in molecular cloning and STR characterization; Pedro Lopes for mycoplasma analysis; José Furtado for the invaluable insights in cell culture; Luís Vieira for the fruitful discussions about qPCR strategies; and Juliane Menezes for qPCR training.

Funding

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Ethical statement

Ethical approval for the study was granted by the institutional review boards of INSA, in line with the principles of the Declaration of Helsinki.

4.6. SUPPLEMENTARY MATERIAL

Supplementary Table S4.1. RT-qPCR primers

Target (vector or gene)	Primer	Primer Sequence (5'-3')
VCAM1	Fw	TGG ACA TAA GAA ACT GGA AAA GG
	Rv	CCA CTC ATC TCG ATT TCT GGA
NOS3	Fw	GGC ATC ACC AGG AAG AAG ACC
	Rv	TCA CTC GCT TCG CCA TCA C
HMOX1	Fw	ATG GCC TCC CTG TAC CAC ATC
	Rv	TGT TGC GCT CAA TCT CCT CCT
GAPDH	Fw	AGC CAC ATC GCT CAG ACA C
	Rv	GCC CAA TAC GAC CAA ATC C

4.6.1. *Mycoplasma* Testing of Cell Cultures

Cell lines were mycoplasma-tested using an in-house protocol. Cell lysates for mycoplasma detection were prepared from 1 mL of supernatant medium, collected at cell confluency, and at least three days after trypsinization. The cell pellet was resuspended in lysis buffer (50 mM Tris HCl; 0,5% Triton X-100) and proteinase K (0,5mg/mL), and incubated for 15 min at 37ºC followed by 10 min at 95ºC. Touchdown PCR (Bio-Rad, C1000, CA, USA) was performed on cell lysates using the following primer sequences: (Forward) 5'-TGCACCATCTGTCACTCTGTTAACCTC-3', (Reverse) 5'-ACTCCTACGGGAGGCAGCAGTA-3'. These amplify an approximately 700bp fragment from the 16S ribosomal DNA region from the bacterial class Mollicutes. PCR products were subjected to agarose gel electrophoresis and visualized in a gel imaging system (UVITEC Cambridge, FireReader, Cambridge, UK). The results indicated an absence of amplification of the bacterial fragment, indicative of mycoplasma-free cultures of HBECs as well as EA.hy926 cells.

4.6.2. STR Analysis for Cell Line Authentication

4.6.2.1. DNA Extraction

One 25 cm2 tissue culture flask, with at least 70% confluence, was used to harvest cells for DNA extraction according to standard procedures. DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega, Madison, USA) according to the manufacturer´s instructions.

4.6.2.2. STR Amplification and Detection

A multiplex PCR for the detection of eight STRs (D7S820, CSF1PO, TH01, D13S317, D16S539, vWA, TPOX, D5S818) and Amelogenin was performed using 1ng of DNA and the AmpFlSTR Identifiler PCR Amplification kit (Applied Biosystems, Warrington, UK) according to the manufacturer´s instructions. STR fragment detection was performed by capillary electrophoresis in an ABI3500 Genetic Analyzer (Applied Biosystems, Foster City, USA). Allele size analyses were carried out with GeneMapper v6.0 (Applied Biosystems, Foster City, USA). The ATCC and DSMZ STR databases [430,431] were used for confirmation of the identified alleles.

For HBECs, the result of the comparison between STR profiles of the test and reference samples was in the range of 80-100% using both databases, consistent with the two samples being related (same donor). A similar result was obtained for the STR profiles comparison between the EA.hy926 cell test and reference samples.

4.6.3. Karyotype Analysis

Karyotype analyses were carried out at passages 2-3. Cell harvesting and chromosome banding were performed using a modification of standard *in situ* methods. Briefly, cells were synchronized with Chromosome Resolution Additive

(ProCell InCulture, Alpha Metrix Biotech GmbH, Rödermark, Germany), according to manufacturer's instructions, before colcemid treatment. Chromosome preparations were obtained using the standard GTL-banding method. Karyotype analyses were performed using a CytoVision® automated imaging system (Leica Biosystems, Nussloch GmbH, Germany). A total of 40 cells distributed by three independent cultures were analyzed for the determination of the chromosome modal number.

EA.hy926 cells had a modal number of 73 chromosomes (range 42-75), reflecting a near-triploid female complement. Karyotype analysis of HBECs showed a near-diploid male complement, with a modal number of 44 chromosomes (range 40-85). Chromosome abnormalities observed in both cell lines are included in Supplementary Table S4.2.

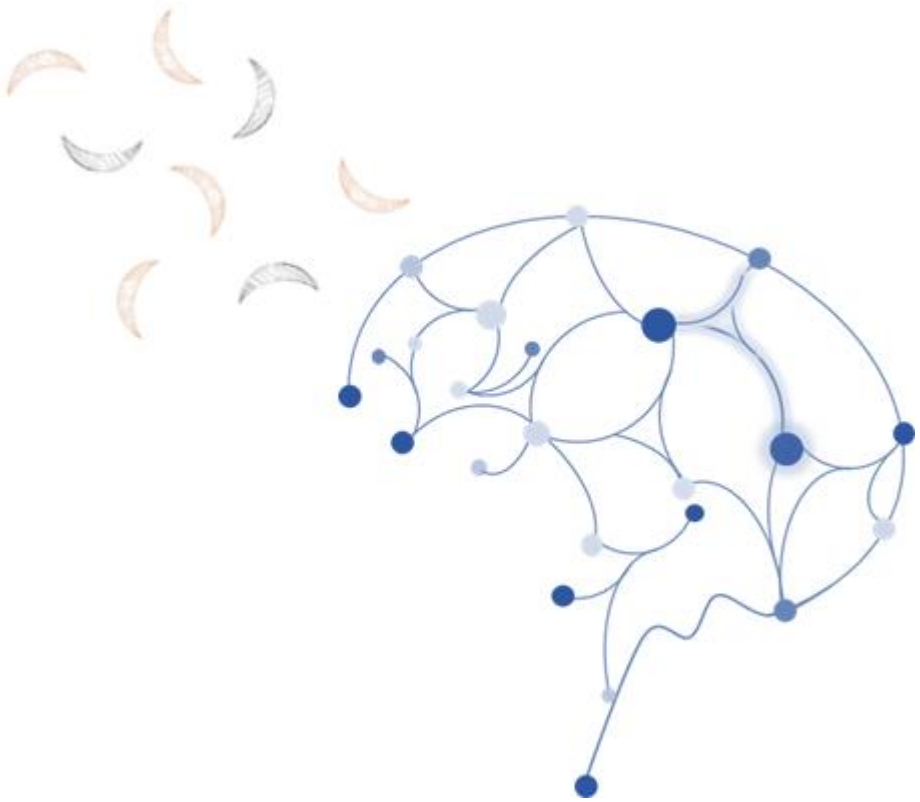
Supplementary Table S4.2. Chromosome abnormalities observed in EA.hy926 and HBEC cell lines

Chromosome	EA.hy926	HBEC
1	del(1)(q10) der(1)(q10;p10) der(1)t(1;14)(p10;q10) der(1)t(1;3)(p10;p10)	der(1)t(1;6)(p10;q10)
2	der(2)t(2;14)(p10;q10) der(2)t(2;3)(q10;q23)	-2 del(2)
3	i(3)(p10) t(3;7)(p10;q10)	dic(3;22)(p25;p11.2)
4		del(4)(q10)(q21q28)
5		dic(5;11) i(5)(q10)
6	der(6)t(6;8)(q10;q10) der(6)t(6;9)(q27;q12)	t(6;9)(q25;p24)
7	del(7)(q11.2)	del(7)(q11.2)* del(7)(p12p15) der(7)t(7;22)(p22;q10)

8	der(8)t(8;21)(q10;q10) der(8)t(1;8)(q23;q24)	
9		del(9)(q11.2) der(9)t(X;9)(q15;p24) der(9)t(3;9)(p21;p24) der(9)t(8;9)(q23;p10) der(9)t(9;22)(q10;q10)
10		-10
11	del(11)(q23.3) +11	-11 der(11)t(11;13)(q10;q10) dic(11;16)(q25;q24)
12	del(12)(q24.2) der(12)t(2;12)(q32.2;p11.2)	
13	der(13)t(13;17)(q32;q21.3)	-13 der(13)t(5;13)(p10;q10) der(13)t(10;13)(q10;q10) der(13)t(13;14)(q10;q10) der(13)t(13;15)(p10;q10) der(13)t(13;18)(q10;q10) i(13)(q10)
14	der(14)t(2;14)(p13;q10) i(14)(q10)	-14
15	der(15)t(15;19)	der(15)t(15;21)(q10;q10) der(15)t(15;22)(q10;q10)
16	+16	-16 del(16) der(16)t(16;21)(q10;q10)
17	+17	-17
18	+18	
19	der(19)t(X;19)(p22;q13.1)	
20	+20,+20 der(20)	-20 dup(20)(q11.2q13.3)
21	der(14;21)(q10;q10)	-21
22	der(22)t(8;22)(p11.2;q10)	-22
X	del(X)(q25)	dic(X)t(X;9)(p22.1;p24)* del(X) der(X)t(X;10)*

		der(X)t(X;5;11)
Y	-	-Y dic(Y;13)(p11.2;p11.2)
other	sSMC	sSMC

Only abnormalities observed in more than one cell are included; *supernumerary; **sSMC**: small supernumerary (satellited) marker chromosome



Concluding Remarks and Future Perspectives

Part IV

Chapter 5

CONCLUDING REMARKS

5. OUTLINE OF PREVIOUS CHAPTERS

Three scientific studies, with implications for SCA cerebral vasculopathy pathophysiology, were presented in this dissertation. Introductory **part I** provided context and a review of the current knowledge of SCA in general and pediatric vasculopathy in particular, followed by the objectives of the project. **Part II** described two studies, with an assessment of putative biomarkers and genetic modulators of pediatric cerebral vasculopathy in SCA (**chapter 2**), and an *in silico* prediction of the potential functional consequences of the most significant genetic modulators identified (**chapter 3**). **Part III** included, in **chapter 4**, a functional approach, using microvascular and macrovascular EC models, to confirm modulation of *VCAM1* haplotypes identified with a significant association with stroke and stroke risk. This approach took advantage of a comparison, in the same EC models, of *VCAM1*, *HMOX1,* and *NOS3* expression studies after pro-inflammatory and pro-oxidative stimulation. Pharmacological rescue with hydroxyurea was also tested.

Part IV includes the present chapter and chapter 6. The current chapter comprises a summary of the main findings of each section, followed by a general discussion where those findings are reviewed and the chapters analyzed through a broader perspective. Finally, **chapter 6** concludes with some recommendations regarding further studies and future perspectives.

5.1. MODULATION OF PEDIATRIC CEREBRAL VASCULOPATHY IN SCA

In **chapter 2**, we assessed demographic, clinical, biochemical, hematological, genotyping and imaging data to evaluate the possibility of designing a biomarker panel for cerebral vasculopathy prognosis in children with SCA. With this approach, we were

able to identify statistically significant associations between biochemical, as well as hematological parameters, with genetic variants and cerebral vasculopathy.

The genetic variants with stronger potential effect in cerebral vasculopathy modulation were the *VCAM1* rs1409419_T allele and promoter haplotype 7, as well as the *VCAM1*-ligand gene *ITGA4* rs113276800_A and rs3770138_T alleles, which showed positive association either with stroke, stroke risk or with cerebral vasculopathy globally. As with the other *VCAM1* promoter variants, the presence of the rs1409419_T main consequence would be the change in transcription factor binding site(s). All the transcription factors whose expression may be affected by the significantly associated variants are mainly involved in development and in different tissues, following the proposed role of VCAM-1 [110]. Another important point regarding *VCAM1* is that even if we address the potential effects of the individual *VCAM1* variants' genotypes, the most important modifying role on disease manifestation would probably arise in the context of the haplotypes that encompass them. The one exception seems to be rs1409419_T given the overlapping findings observed for this variant and haplotype 7, which includes it.

The fact that the rs113276800_A and rs3770138_T *ITGA4* alleles were associated both with stroke and cerebral vasculopathy as a whole, further underlines the role of (sickle RBC and WBC) cell-endothelium adhesion in severe cerebral vasculopathy. We also identified the presence of a not previously described haplotype, constituted by variants rs1375493, rs35723031, and rs10562650, that despite being very frequent in the group of patients studied, was positively associated with lower LDH levels, and potentially with a protective effect against hemolysis.

The *NOS3* variants which were associated with cerebral vasculopathy, also showed a putative protective effect, in this case specifically for SCIs or cerebral vasculopathy

globally. Like *VCAM1* this occurred as a result of the presence of isolated variants (intron 4 VNTR_4b allele with a negative association with SCI) or haplotypes (Haplotype V with a negative association with cerebral vasculopathy globally). An indirect protective effect would be also expected, due to the presence of rs2070744_C and its association with lower RDW values. The latter was recently suggested to be a biomarker for lower cerebrovascular disease risk [371].

We also evaluated other previously identified stroke-associated candidate gene variants – *GOLGB1* rs3732410_G (Y1212C), *ENPP1* rs1044498_C (K173Q), and *PON1* rs662_C (Q192R) [342]. Of the three variants, only *ENPP1* rs1044498 AA and AC genotypes showed a positive association with stroke risk. As for the *PON1* rs662_C variant, albeit no association with stroke or global cerebral vasculopathy was apparent, we observed a positive association with high platelet levels, indicating a potential impact on hemostasis and inflammation.

The only consensual modifiers of SCD disease severity, so far, have been the persistence of HbF beyond infancy and the presence of -$\alpha^{3.7kb}$–thal deletion. The co-inheritance of -$\alpha^{3.7kb}$–thal and homozygous HbS mutation has been associated with an overall ameliorating effect on anemia, particularly a protective effect against stroke in children [151]. We did not find any direct relationship between the presence of the -$\alpha^{3.7kb}$–thal deletion and stroke, although we did find that patients with α–thal showed a higher RBC count, lower MCV, and MCH, consistent with previous reports [374,375]. Other authors have also reported an absence of association between -$\alpha^{3.7kb}$–thal presence and stroke protection [376,377].

Small sample size, population heterogeneity (different Sub-Saharan countries ancestry), or other specific population characteristics were limitations for extrapolation to the general SCA pediatric population. Nevertheless, our results seem to reinforce

the importance of genetic modulators in the pathophysiology of pediatric cerebral vasculopathy and provide clues for the discovery of novel targets for SCA therapy. Our findings suggest that a comprehensive biomarker panel that includes biochemical, hematological, imaging as well as genetic data may be very important for cerebral vasculopathy prediction, and prevention. Even though the patients we studied are subjected to environmental factors (both physical and social) different from those of their ancestry, the genetic modifiers described in our study, namely *VCAM1* haplotype 7 and rs1409419, may provide further tools for cerebral vasculopathy prevention in SCA.

5.2. PREDICTED EFFECTS OF MODULATORS IN SCA

As stated earlier the major and consensual genetic modifiers of SCD clinical manifestations are the co-inheritance of α-thalassemia and those affecting the HbF expression. The work described in chapter 2 allowed us to identify other putative genetic modulators of pediatric cerebral vasculopathy in SCA.

In **chapter 3**, the results of *in silico* analyses of *VCAM1* rs1041163_C, rs1409419_T and rs3917025_delCT promoter variants, as well as *NOS3* coding SNP rs1799983, provide some clues about possible functional roles of these genetic variants in the pathophysiology of SCA, emphasizing their impact on SCA hemorheology. The three promoter variants have the potential for affecting *VCAM1* expression regulation, namely as a result of differences in transcription factor affinity to the altered sequence as compared to the wild-type sequence. Transcription factor binding site changes were indeed observed for the three polymorphic regions.

VCAM1 rs1041163_C was predicted to cause a substitution of the site for a retinoid X receptor factor (RXRF) by one for a PR domain zinc finger protein 1 (PRDM1) which is

a transcription repressor that promotes hematopoietic B cell differentiation and pro-inflammatory cytokine secretion [397]. Therefore in a pro-inflammatory environment, such as an activated endothelium, the presence of this variant might lead to an increase of *VCAM1* inducible expression. In the case of rs1409419_T, it was shown to lead to a potential gain for a binding site for EVI-1, Oct1, or BarX2 transcription factors. EVI-1 is a complex multifunctional that modulates multiple processes, including cell migration, motility, adhesion, response to oxidative stress, proliferation, and apoptosis/survival [364]. Of special interest is the ability to cooperate with a FOS transcription factor to limit cell adhesion while enhancing cell proliferation, one hallmark of oncogenesis [364]. On the other hand, Oct1 is a transcription factor known to promote a transcriptional repression/silencing effect which would potentially lead to *VCAM1* down-regulation. BarX2 has been shown to promote murine muscle cell differentiation by interacting with muscle regulatory factors (MRFs) [399], a gain of which could result in upregulation of gene expression in muscle tissue. Finally, a gain of a FAST1 binding site was identified for rs3917025_delCT. FAST1 is a transcription factor involved in patterning and development of embryonic structures in vertebrates, in a complex network of activation/repression mechanisms [400].

In summary, all transcription factors affected by the promoter variants are mainly involved in development (including in early embryonic stages, as FAST1) and in different tissues, which aligns with the VCAM-1 proposed role in development with tissue- and time-specific expression patterns [110]. In terms of endothelial environment, for instance, one might expect that altered expression levels may affect sickled RBC/EC adhesion as well as endothelium inflammation/activation, thus contributing to endothelial dysfunction and ultimately to impaired blood flow/shear rate.

Even though the *NOS3* rs1799983_G leads to an amino acid change (aspartate for glutamate) in the protein sequence (p.Glu298Asp), all the analyses indicate that this variant is most probably a non-deleterious, nonsynonymous tolerant single nucleotide variant. The apparent conservative (negatively charged) amino acid substitution that results therein would also be in agreement with that observation. Nevertheless, it occurs in the sequence encoding the oxygenase domain of eNOS, which is critical for the enzyme activity and contains both the catalytic site and the components for the oxygenase function. This could potentially lead to impairment of the oxygenase function and thus contribute to higher oxidative stress through decreased heme-binding, eNOS uncoupling, and ultimately to NO bioavailability. Possible alterations in the endothelial location inside the caveolae have also been proposed [396].

Overall, these alterations may affect key points in endothelial dysfunction and/or vascular tone and consequently modulate cardiovascular risk in SCA [401]. Moreover, impaired oxygenase activity would expectedly result in increased oxidative stress thus (i) decreasing healthy RBC deformability along with increasing the strength of RBC aggregates, and (ii) possibly inducing an exacerbated response sickle RBC response, accompanied by a highly abnormal hemorheological profile [reviewed in [402]]. Altered oxygenase function may also impact therapeutic approaches. For instance, it is known that drugs interfering with the renin-angiotensin-aldosterone system, as well as statins, are useful in preventing endothelial dysfunction. However, the mechanisms through which they promote eNOS uncoupling, in the case of elevated oxidative stress, may provide useful clues as to ways of increasing NO beneficial actions in the cardiovascular system [78].

Despite the limitations of the tools, namely input data format, choice of algorithm, cut-off values, and database quality, *in silico* approaches constitute a useful preliminary

step in evaluating the possible biological and clinical consequences of the presence of genetic variants. The information provided, especially the one regarding the *VCAM1* promoter variants served as the basis for the following functional and expression studies.

5.3. FUNCTIONAL STUDIES

Given the strong association of specific *VCAM1* promoter haplotypes with stroke/stroke risk and hemolysis, in the present as well as in our team's past projects [354,382], we set out to assess its functional consequences. In **chapter 4** we describe the modulation effects of *VCAM1* promoter haplotypes 1, 3 and 7, on macrovascular as well as brain microvascular ECs, under a pro-inflammatory stimulus. To evaluate the impact of that modulation we also studied the effect of TNF-α and hemin stimulation in otherwise naïve cells, using the same EC models. Finally, we analyzed if HU treatment provided pharmacological rescue in the latter pro-inflammatory and pro-oxidative context. For comparison purposes, our assessment included *NOS3* and *HMOX1* gene expression under the same stimuli and HU treatment.

Our results showed that the presence of the stroke-associated Hap_ 7, in TNF-α-stimulated ECs led to markedly higher *VCAM1* promoter activity, especially in cerebral microvascular ECs. This will potentially upregulate *VCAM1* expression in cytokine-activated endothelia, namely in the brain. This would have a considerable impact on cerebral vasculopathy in SCA and aligns with our initial findings, described in chapters 3 and 4. The differential response to both Hap_4 and Hap_7, observed in macrovascular and cerebral microvascular ECs, further suggests that these genetic modulators may be critical for the (systemic) vasculopathy phenotype typical

in SCA, and not only for brain vasculopathy. Moreover, and despite *VCAM1* activation is mainly under transcriptional control, the *in silico* studies indicate that neither of the variant alleles composing the studied haplotypes would affect the NF-κB sites on the *VCAM1* promoter [382,415]. The potential gain of EVI-1, Oct1, or BarX2 transcription factor binding sites associated with rs1409419_T indicated by those studies does not directly explain the *VCAM1* upregulation we observed, as these transcription factors individually usually work as negative regulators of gene expression [416,418,432]. However, we cannot exclude a cooperation between these transcription factors and the two TNF-α sensitive NF-κB sites.

We also observed that brain microvascular ECs showed a baseline *VCAM1* expression that was further increased after TNF-α stimulation, while macrovascular ECs only expressed *VCAM1* after stimulation. The opposite effect was found for *NOS3* expression, which was downregulated by TNF-α, namely in brain microvascular ECs. This effect was only counteracted by treatment with the highest HU concentration. Cerebral ECs actively participate in central nervous system (CNS) inflammation, due to their proximity to astrocytes, pericytes, neuronal cells, and perivascular microglia [reviewed in [425]]. They express TNF-α receptors TNFR1 and TNFR2, through which they can modulate endothelial tight-junctions and cytoskeleton rearrangement. Moreover, they have been shown to regulate iNOS and ICAM-1 production by an autocrine TNF-α-dependent pathway, as a response to infection [426]. The ability to produce iNOS in response to external stimuli, hence providing an alternative source of NO, may explain the marked downregulation of *NOS3* expression we found in HBECs. We did not observe increased *VCAM1* expression in macrovascular ECs after hemin exposure, and in cerebral microvascular ECs the effect was lower than the one

induced by TNF-α. This is in line with previous reports of hemin-associated decreased VCAM-1 expression and NF-κB activation in the lungs, liver, and spleen of sickle mice [427], due to hemin-induced HO-1 upregulation, and with the absence of HbS to which ECs of SCA patients are usually exposed to [158]. *HMOX1* hemin-induced upregulation was also apparent in our experiments and with a similar degree in both EC models. Thus there seems to be little difference in the levels by which this antioxidant system operates in the macrovascular and brain microvascular environments. Conversely, we observed a marked hemin-induced *NOS3* downregulation in HBECs which may also occur as a result of the alternative iNOS activation described earlier.

HU modulation on gene expression was assessed by treating ECs with two different HU concentrations – 250 μM and 46 mM. The 250 μM HU treatment was based on plasma concentrations observed in patients treated with HU therapeutic dosages [429], while the HU 46 mM concentration was based on the maximum tolerated dose of 35 mg/kg/day recommended for pediatric patients [413], adjusted to human body density. Moreover, we combined HU treatment with hemin at 70 μM, to assess EC response in pro-oxidative conditions, based on recent reports [317,414]. The modulating effect of HU on *VCAM1* expression, in both EC types, was limited by the pro-inflammatory and pro-oxidant endothelial context. In neither of the two dosages did the drug reduce *VCAM1* mRNA relative levels below baseline values. The impact was even lower in hemin-treated ECs. This suggests that HU treatment may not suffice to lower stroke risk in patients with the *VCAM1* haplotype 7 and rs1409419_T allele, given their predicted impact on cerebral microvascular ECs.

Differential HU modulation also occurred for *NOS3* but in a dose-dependent manner, which was consistent with observations by da Guarda and colleagues [411]. Only the concentration equivalent to the maximum recommended dose in the pediatric SCA

setting (46 mM) was associated with increased expression, namely in brain microvascular ECs. This suggests that the beneficial HU effect on NO levels may not be directly on *NOS3* expression, and is more likely post-translational, as the proteasomal protection from degradation mechanism proposed by other authors [310,411]. Hence it is possible that HU may enhance the protective effect on SCI and/or cerebral vasculopathy we previously identified associated with intron 4_27bp VNTR allele 4b (5 repeats), haplotype V (rs2070744_T / VNTR_4b / rs1799983_G) and haplotype VII (rs2070744_T / VNTR_4c / rs1799983_G). However, this “cooperative” effect was dependent on the HU dose administered. We found no effect of HU on *HMOX1* expression, either in EA.hy926 cells or HBECs, which reinforces the hypothesis of Santana and colleagues that *HMOX1* follows an alternative mechanism of antioxidant response not susceptible to HU action [317].

Our study did not address the possibility of post-translational modification, the absence of surrounding tissues in our models, and the effect of HbS and sickle RBCs on vascular ECs. Despite these limitations, our observations further reiterate the differential modulation of endothelial cell expression at the level of adhesion molecule production, NO production, and cytoprotective antioxidant systems. Moreover, and despite the overall benefits of HU in several SCA manifestations, there are still pathophysiological mechanisms that are not susceptible to its disease-modifying effects.

5.4. Concluding Remarks

The main goal of our research project was to increase the knowledge of the pathophysiology underlying pediatric cerebral vasculopathy in SCA. For that purpose, we focused on the genetic modulation of cerebral vasculopathy severity.

Building on previous results of our research team, we started by using a candidate gene approach, in a group of pediatric SCA patients with Sub-Saharan ancestry, well-characterized in terms of cerebral vasculopathy. The population studied was enrolled by the major hematology centers that treat pediatric SCA patients in Portugal.

The candidate gene approach was further expanded by assessing potential cerebral vasculopathy association not only with genetic variants but also with biochemical and hematological data. We found that specific genetic variants in *VCAM1* and *ITGA4* genes were associated with stroke, reflective of the crucial role of cell-endothelium adhesion in cerebral vasculopathy pathophysiology. Conversely, *NOS3* variants were predicted to exert a protective effect on SCI and cerebral vasculopathy globally, which is consistent with the NO importance in vascular function. Conversely, we could not confirm associations between stroke or stroke protection and modifiers suggested in other studies, like *GOLGB1*, *PON1*, or the most consensual $-\alpha^{3.7kb}$ deletion. As expected, lower HbF levels were positively associated with stroke and stroke risk. Reduced MCH showed a similar effect on stroke, while high neutrophil and platelet counts, as well as high LDH levels, were positively associated with stroke risk.

Although the size and heterogeneity of the study population limited global extrapolations to the general pediatric SCA population, we confirmed the potential of genetic modulation on cerebral vasculopathy and obtained clues as to how that modulation may occur in those patients.

The next step of our project was to evaluate *in silico* the predicted effect of the variants initially identified, before initiating the biological assays. Since polymorphisms on the *VCAM1* promoter stood out in the previous study we focused on those variants, and also on a coding *NOS3* variant, analyzing their potential consequences at the

hemorheological level. We found potential transcription factor binding site changes as a result of the presence of the *VCAM1* promoter variants studied. A special note for rs1409419_T which was predicted to lead to an EVI-1, Oct, or BarX2 binding sites. In isolation, these transcription factors have been described as having a negative regulatory effect on gene expression. EVI1 especially was previously described to act on infection-induced NF-κB activity in a negative feedback manner to regulate inflammatory response. However, in neoplasia, this transcription factor may also cooperate with others to inhibit adhesion but induce cell proliferation. Since this mechanism of cellular proliferation is also a key aspect in the pathophysiology of SCA vasculopathy, it is reasonable to assume that a gain of an EVI-1 transcription factor binding site may underlie the rs1409419_T (and Haplotype 7) impact on stroke and stroke risk. On the other hand, and notwithstanding its location on the enzyme's oxygenase domain sequence, the *NOS3* coding variant rs1799983_G was not predicted to have direct functional consequences. This finding was in line with the absence of association with the cerebral vasculopathy we observed in the genetic association studies.

Both the expanded genetic association and *in silico* studies led us to choose the *VCAM1* promoter haplotypes for further investigations. Hence we designed functional studies that would assess the promoter's activity in the presence of Haplotype 7 (stroke-associated) or Haplotype 4 (hemolysis-associated). To contextualize the results we also evaluated endothelial *VCAM1* expression, simulating pro-inflammatory and pro-oxidative stimuli the macrovascular and cerebral microvascular ECs are exposed to in SCA. We added to the study the expression analyses of two other genes (*NOS3* and *HMOX1*), for comparison purposes. Our results confirmed the upregulation effects of both haplotypes on *VCAM1* promoter activity, with the stroke-associated

Haplotype 7 having a stronger effect on brain microvascular ECs. Furthermore, the expression studies indicated that *VCAM1* expression occurs in naïve cerebral microvascular ECs, and highly increases after cytokine stimulus. This upregulation was apparently counteracted by HU treatment, however never to baseline levels. This seemingly insufficient pharmacological rescue would potentially impact stroke prevention in pediatric patients with the risk haplotypes. The *VCAM1* upregulation observed also in macrovascular ECs due to the presence of the promoter haplotypes studied, namely Haplotype 4, suggests that these haplotypes may have implications, not only on cerebral but also on systemic vasculopathy.

While the expression studies of *NOS3* also confirmed differential EC expression, *HMOX1* expression was similar in both EC models. Additional differences include the response to HU treatment. HU upregulation of *NOS3* was dose-dependent, whereas *HMOX1* expression seemed impervious to HU action.

Even though extrapolation for pediatric vasculopathy and specific organ-damage mechanisms should be done with care and after corroboration with further ex-vivo studies (using patient biological samples), overall our results reinforce the importance of *VCAM1* in endothelial cell response in SCA. The fact that the variants studied affect a regulatory region is of considerable significance since *VCAM1* activity is mainly controlled by transcriptional regulation. Being frequent in children of Sub-Saharan ancestry with SCA, they may impact how these patients respond to stroke prevention strategies, and ultimately for devising therapies for HU-non-responsive patients. Further knowledge of the mechanisms by which the differential modulation occurs is crucial. The impact on SCA is of particular importance for the more recent multi-agent therapeutic approaches.

Chapter 6

FUTURE PERSPECTIVES

6. FUTURE PERSPECTIVES

As discussed earlier, the current stroke prevention strategies are insufficient to identify all pediatric SCA patients at risk of cerebral vasculopathy. Therefore, additional biomarkers are essential to provide alternatives and to help develop more accurate and comprehensive risk assessment approaches. Moreover, it is important to devise personalized pharmacological cocktails that address patient-specific complaints.

The results obtained in the present work provided new clues in the role of biomarkers in pediatric cerebral vasculopathy. We believe that they can be complemented by further research efforts.

Multicenter and multidisciplinary cooperative studies would allow the evaluation of the modulators here identified in larger numbers of SCA pediatric patients. As discussed, it is important to thoroughly characterize those SCA patient populations with imaging, biochemical and hematological data. Sample size, population heterogeneity, and patient ancestry are key elements, in addition to experienced specialists and a carefully planned study design, would ensure efficient patient enrolment in terms of numbers and population characteristics.

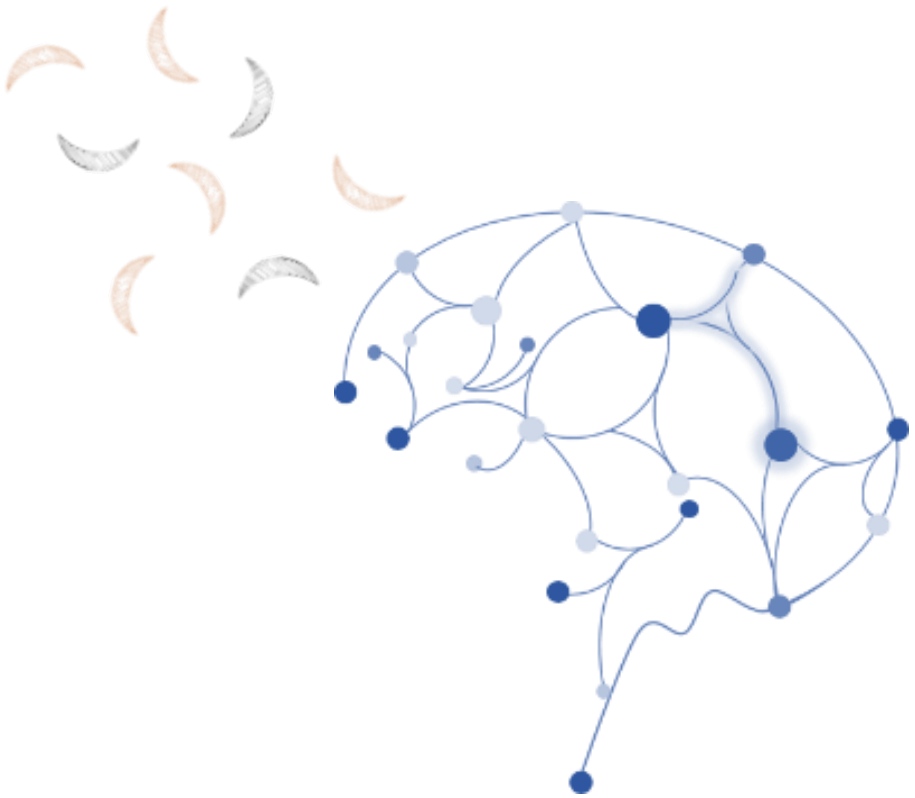
Extending the gene expression studies we performed to samples of pediatric patients with SCA, namely children with the potential susceptibility *VCAM1* haplotypes, would be essential not only for confirmation purposes but also to assess the mechanisms by which phenotype modulation may occur. If altered *VCAM1* expression is confirmed the potential use of the modifier haplotypes/variant as novel genetic biomarkers of disease prognosis is an added benefit. In this study, we initially planned to establish fibroblast cell cultures from skin explants of pediatric patients with the *VCAM1* promoter haplotype 7, and subsequently perform the RT-qPCR studies to assess *VCAM1* expression changes. Unfortunately, those efforts were hindered by the ongoing

COVID-19 pandemic context. Analyses of transcription factor binding (e.g., by chromatin immunoprecipitation) would also potentially provide useful insights regarding the mechanisms by which the presence of haplotypes 4 and 7 affect the *VCAM1* promoter.

Adhesion studies would also be valuable to evaluate the effects of *ITGA4* variants on sickle cell adhesion to the endothelium. This could be achieved through biochip-based assays, using blood samples collected from patients. Those microfluidic platforms allow simulations of the blood flow conditions and shear stress that occur in SCA patients' blood vessels, measure the adhesion and the flow rate observed. Vaso-occlusive processes could be simulated through this strategy as well. Homotypic and heterotypic cell interactions, specifically those involving sickle cells and leukocytes, could be studied using imaging flow cytometry.

On the other hand, besides *ex vivo* expression studies, assessing the *NOS3* predicted protective effect on cerebral vasculopathy would also require, indirect approaches that rely on the measurement of eNOS end-products (e.g. nitrite accumulation).

In summary, a wide array of research possibilities have arisen as a result of this research project which would complement it, and be beneficial in providing therapeutic targets and contribute to pediatric cerebral vasculopathy prevention in SCA.



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Part V

Chapter 7

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7. REFERENCES

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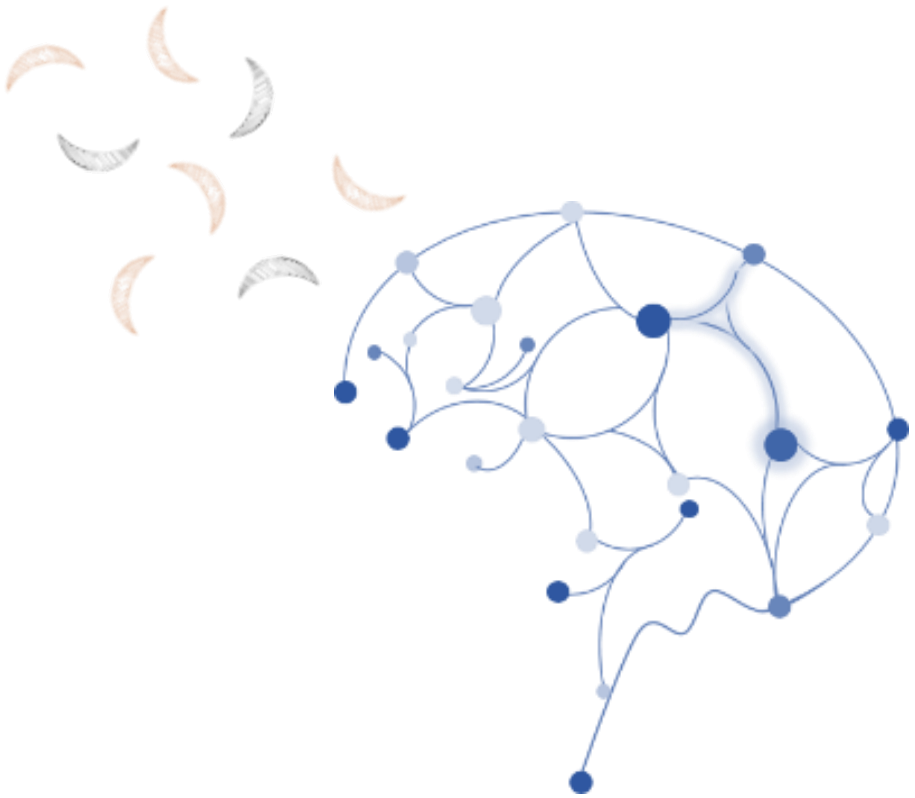

Appendices

Part VI

Chapter 8

APPENDICES

8.1. APPENDIX A

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Biomarkers and genetic modulators of cerebral vasculopathy in sub-Saharan ancestry children with sickle cell anemia

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ABSTRACT

We investigated biomarkers and genetic modulators of the cerebral vasculopathy (CV) subphenotype in pediatric sickle cell anemia (SCA) patients of sub-Saharan African ancestry. We found that one *VCAM1* promoter haplotype (haplotype 7) and *VCAM1* single nucleotide variant rs1409419_T were associated with stroke events, stroke risk, as measured by time-averaged mean of maximum velocity in the middle cerebral artery, and with high serum levels of the hemolysis biomarker lactate dehydrogenase. Furthermore, VCAM-1 ligand coding gene *ITGA4* variants rs113276800_A and rs3770138_T showed a positive association with stroke events. An additional positive relationship between a genetic variant and stroke risk was observed for *ENPP1* rs1044498_A. Conversely, *NOS3* variants were negatively associated with silent cerebral infarct events (VNTR 4b_allele and haplotype V) and CV globally (haplotype VII). The -alpha$^{3.7kb}$–thal deletion did not show association with CV. However, it was associated with higher red blood cell and neutrophil counts, and lower mean corpuscular volume, mean corpuscular hemoglobin and red cell distribution width.

Our results underline the importance of genetic modulators of the CV sub-phenotype and their potential as SCA therapeutic targets. We also propose that a biomarker panel comprising biochemical, hematological, imaging and genetic data would be instrumental for CV prediction, and prevention.

1. Introduction

Sickle cell anemia (SCA), the homozygous form of the c:20A > T mutation in the beta-globin gene, is the most common and severe presentation of sickle cell disease (SCD). High birth rates and child mortality are most frequent in sub-Saharan Africa, however, population movements are leading to a wider distribution, emphasizing the global health emergence status of the disease [1–3]. In children, the most common sub-phenotypes are cerebral vasculopathy (CV), acute chest syndrome, hyposplenism, renal disease and painful crises. CV is a major complication and comprises overt stroke, silent cerebral infarcts (SCIs), transient ischemic attacks and frequently neurocognitive complications

Abbreviations: SCA, sickle cell anemia; SCD, sickle cell disease; CV, cerebral vasculopathy; SCI, silent cerebral infarction(s); LDH, lactate dehydrogenase; TAMMV, time-averaged mean velocity in the middle cerebral artery; TCD, transcranial Doppler ultrasound; MRI, magnetic resonance imaging; MRA, magnetic resonance angiography; HC, hydroxycarbamide; HbS, hemoglobin S; HbF, fetal hemoglobin; Hb, total hemoglobin; RBC, red blood cells; WBC, white blood cells; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; RDW, red cell distribution width; NGS, next-generation sequencing; VCAM-1, vascular cell adhesion molecule 1; ITGA4, very-late antigen 4; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; TFBS, transcription factor binding site(s)

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at a later stage. Children with SCA have a much higher risk of stroke than the general pediatric population. The prevalence of overt stroke approaches 11% by age 20 years. On the other hand, SCIs have been found in up to 37% of children with SCA [4].

The current standard of care for stroke prevention in SCA children is Transcranial Doppler (TCD) screening – through measurement of time-averaged mean velocity (TAMMV) in the middle cerebral artery – followed by regular blood transfusions therapy and hydroxycarbamide (HC) treatment. Despite its high sensitivity, TCD still does not allow identification of all SCA children that will experience a stroke and, conversely, children with high TAMMV (> 200 cm/s) may not develop stroke [5]. Moreover, blood transfusion/HC therapies are not without limitations or adverse side effects [6]. On the other hand, although diagnosis with magnetic resonance imaging, MRI, (or angiography, MRA) is recommended for early diagnosis of SCIs and recognition of large vessel stenosis, MRI/MRA are not useful to identify patients at risk of developing SCIs or large vessel stenosis [7]. A more specific and sensitive panel of biomarkers for CV prediction and prognosis, that includes genetic variants with disease modifying effects, is therefore of the utmost importance.

In previous studies, we identified variants in *VCAM1*, *NOS3* and *HBA* with a positive association with chronic hemolysis, a known pathophysiological SCA mechanism [8]. Building on those results we aimed, in this work, to assess if those variants were also associated with pediatric CV in a sub-Saharan SCA population. Our candidate gene approach also focused on the VCAM-1 ligand gene, *ITGA4*, and for comparison purposes, we included the three genetic variants (*PON1* rs662, *ENPP1* rs1044498 and *GOLGB1* rs3732410) previously reported in association with pediatric stroke in SCA patients [7,9,10].

2. Materials and methods

2.1. Ethical statement

Ethical approval for the study was granted by the institutional review boards of Instituto Nacional de Saúde Dr. Ricardo Jorge (INSA) and of participant hospitals, in line with the principles of the Declaration of Helsinki. The aim and study procedures were explained to the children's parents (or legal guardians) and they provided informed written consent prior to their enrolment in this study.

2.2. Study population

This case-control study was performed at INSA in cooperation with four hospitals in the Greater Lisbon area – Hospital D. Estefânia, Hospital de Santa Maria, Hospital Fernando Fonseca and Hospital Garcia de Orta, the four largest centers of that metropolitan area. These centers combined receive the highest numbers of SCA pediatric patients in our country. Seventy unrelated pediatric patients (≥3 years old) of direct sub-Saharan African ancestry diagnosed with SCA were selected. Exclusion criteria included age < 3 years old, non-African ancestry, previous HC treatment or having received a blood transfusion in the 120 days prior to enrolment.

Data obtained from patients and parents (or legal guardians) interviews, which included demographic characteristics (age, gender, parents' geographic origin), were collected. Hemoglobin profiles (HbS, HbF), hematological parameters [RBC, leukocyte, neutrophil and platelet counts, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), red cell distribution width (RDW)] as well as hemolysis biochemical and hematological biomarkers [serum LDH and total bilirubin and reticulocyte count] were retrospectively collected from patients' hospital records. All these parameters were obtained by standard procedures and HbF levels, in particular, were measured by high-performance liquid chromatography (HPLC). The data collected for each parameter were the result of, at least, three different time-point measurements, performed in steady-state periods, and prior to any treatment with HC and more than > 120 days after receiving a blood transfusion. The patients were analyzed according to CV outcome, or stroke risk, in the following groups (i) overt ischemic stroke (≥1 episode), henceforth designated as "stroke" ($n = 62$); (ii) "silent cerebral infarct"(≥1 event), SCI ($n = 53$); (iii) stroke and/or SCI, as confirmed by MRI or MRA, ($n = 62$); or "stroke risk" ($n = 60$), with risk stratification provided by the TAMMV values, obtained during TCD, as follows: (a) high risk – TAMMV ≥200 cm/s; (b) conditional (or moderate) risk – 200 cm/s > TAMMV ≥170 cm/s; and (c) low risk – TAMMV < 170 cm/s.

2.3. Genotyping

Genomic DNA was isolated from peripheral blood samples of each patient using the MagNA Pure LC Instrument (Roche Diagnostics GmbH, Mannheim, Germany). All samples were anonymized and specific genotypes could be linked to phenotypes only through the main study database.

The homozygous status for the SCA mutation in the *HBB* gene (c.20A > T) was confirmed by polymerase chain reaction followed by restriction fragment length analysis (PCR-RFLP) with the endonuclease *Bsu*36 I. Beta-globin cluster haplotypes were assigned after examining six restriction endonucleases sites within the cluster: *Xmn* I (5′ to *HBG2*), *Hin*d III (within the *HBG2* and *HBG1*) *Hin*c II (within and 3′ to *ψHBB*) and *Hin*f I (3′ to *HBB*). Aliquots of the amplified products were digested with the appropriate enzymes under the conditions recommended by the manufacturers. Haplotypes were determined by the presence or absence of cleavage at each site and by analyzing the compiled pattern through comparison to known patterns [11]. The $-\alpha^{3.7kb}$–thal deletion was assessed by gap-PCR [12].

Putative modifier genes were identified through previous studies by our group [8] and from other published reports based on the influence on the phenotypes of interest. These candidate genes were used to identify and genotype SNPs and other variants in patient samples. For *VCAM1*, *NOS3*, *PON1*, *ENPP1* and *GOLGB1* genes genotyping was performed using TaqMan-based PCR with commercially available or customized primers.

2.3.1. Screening for ITGA4 variants by next-generation sequencing (NGS)

In order to search for variants in the regulatory region of *ITGA4* gene, NGS analysis was used on a long PCR fragment (4.1 kb), including its flanking regions. PCR was performed using the primers FW5′-CAG AGGCTCATTAGGACCC-3′ and Rv5′-CCTTGCGGTACTATCCAGGC-3′ and the FailSafe enzyme with the PreMix G (Epicentre, Illumina, San Diego).

The NGS workflow consisted in five steps: PCR product purification using paramagnetic beads (Agentcourt, Ampure XP); double-stranded DNA quantification in a Qubit fluorometer; DNA library preparation using the Nextera XT kit (Illumina, San Diego) following the manufacturer's instructions; high throughput sequencing in a MiSeq benchtop sequencer (Illumina, San Diego); data analyses were performed using the following tools: Sequencing Analysis Viewer (v1.8.46, Illumina) and FastQC (v0.11.5, Babraham Bioinformatics,https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) were used for quality analysis. The MiSeq® Reporter software package (v2.6.2, Illumina) was used for read mapping (with Burrows-Wheeler Aligner) and variant calling and filtering (with Genome Analysis Toolkit). FastQ screen (v.0.9.3, Babraham Bioinformatics) was used to screen for contamination between samples. Variant Effect Predictor (www.ensembl.org) was used to annotate variants and Integrative Genomics Viewer (v.2.3.86) (Broad Institute; [13]) was used for visualization of reads and variants. Validation of the variants was performed using automated Sanger sequencing, after amplification with customized primers in the 3130XI Genetic Analyser, (Applied Biosystems). The results were analyzed using FinchTV v.1.4.0 software (Geospiza, Inc.). The genotyping results were added to the previously created database.

2.3.2. Haplotype reconstruction

Haplotype reconstruction was performed using PHASE software v2.1 developed by Mathew Stephens at Washington University, according to the developer's instructions (https://els.comotion.uw.edu/express_license_technologies/phase). Haplotypes were reconstructed for genetic variants in *NOS3* (rs2070744, intron 4_27 bp VNTR, rs1799983), in the promoter of *VCAM1* (rs1409419, rs3917024, rs3917025, rs3783598, rs1041163, rs3783599) and for genetic variants of *ITGA4* (rs1375493, rs35723031, rs10562650; rs1839269 and rs1839268).

2.3.3. In silico analysis

Population allele and genotype frequencies were recorded for each observed variant using the Ensembl browser (www.ensembl.org). SNPs' sequences were retrieved using the NCBI SNP search engine (http://ncbi.nlm.gov/snp).

Transcription factor binding sites (TFBS) analysis was performed for the variants identified in the regulatory regions, using the MatInspector tool (Genomatix, Munich, Germany), to evaluate potential effects on the regulation of gene expression by altering putative TFBS. Only results above the 0.85 threshold were considered, which corresponds to a maximum of 15% dissimilarity between the identified sequence and the consensus sequence. A comparison with previously reported consensus sequences of TFBS for the *VCAM1* [14] and the *ITGA4* [15] promoters was performed. The sequences of the identified variants were not found to overlap with any of the previously reported TFBS consensus sequences. Hence, no strong effects for these genes' expression are to be expected as a result of the presence of those variants.

2.3.4. Statistical analysis

The analyses were performed using the SPSS software (version 25.0, IBM Inc., Chicago, USA). For descriptive analysis, continuous variables were represented as medians and interquartile ranges (IQR). To evaluate the Gaussian distribution of variables, Shapiro-Wilk normality tests where applied. We used the Mann-Whitney *U* test to compare the medians of variables. Categorical variables are represented as number, frequencies and percentages. The chi-square test or the Fisher's exact test were used to compare categorical variables on bivariate analysis. Statistical significance was defined as $p < 0.05$.

The minor allele for each variant was evaluated for potential association with stroke, SCI, cerebral vasculopathy (stroke and SCI combined) or risk (as measured by TCD-TAMMV values), *via* 2 × 2 phenotype × genotype contingency tables. Only polymorphisms with a minor allele frequency (MAF) > 5% were considered for association analysis. Phenotypes were classified as follows: (i) "stroke" (at least one previous overt ischemic stroke event, as confirmed by MRI/MRA) or "no stroke" (no clinically/imaging identified stroke event); (ii) "SCI" (at least one event as identified by MRI/MRA) or "no SCI" (no SCI events, as confirmed by MRI); (iii) "cerebral vasculopathy" (at least one overt ischemic stroke and/or SCI event) or "no cerebral vasculopathy"; and (iv) "risk of stroke" (high/moderate: TAMMV ≥ 200 cm/s or 199 ≥ TAMMV ≥ 170 cm/s; low: TAMMV < 170 cm/s).

Each variant was also evaluated for potential association with biochemical and hematological parameters, including hemolysis biomarkers (LDH, total bilirubin, reticulocyte count).

3. Results

3.1. Population description and genotyping

This study was performed on seventy unrelated SCA patients, (age range: 3–16 years, 40 males, 30 females), living in Portugal but of direct sub-Saharan ancestry, with parental geographic origin mainly from Angola, São Tomé and Príncipe and Cape Verde (Table 1).

Table 1
Demographic, neurological status and laboratory parameters of the population in this study.

Age (years)		3–16	
Gender		n	%
Male		40	57.1
Female		30	42.9
Parental geographic origin		n	%
Angola		42	60.0
São Tomé and Príncipe Islands		8	11.4
Cape Verde		5	7.1
Guinea-Bissau		7	10.0
Guinea-Conakry		1	1.4
Nigeria		1	1.4
Double origin		6	8.6
Neurological status (n = 70)		n	%
Stroke	Yes	15	24.2
(n = 62)	No	47	75.8
SCI	Yes	9	16.9
(n = 53)	No	44	83.0
CV	Yes	24	38.7
(n = 62)	No	38	61.3
Stroke risk	High (TAMMV ≥ 200 cm/s)	25	41.7
(n = 60)	Moderate (170 cm/s ≤ TAMMV ≤ 200 cm/s)	6	10.0
	Low risk (TAMMV < 170 cm/s)	29	48.3
Hematological parameters		Median	IQR
Hb S (%)		79.9	14.5
Hb F (%)		10.7	11.7
Hb (g/dL)		8.0	1.3
RBC ($\times 10^{12}$/L)		3.0	0.7
MCV (fL)		81.3	14.3
MCH (pg)		26.9	6.0
Reticulocytes (%)		9.9	6.3
RDW (%)		21.2	4.5
Platelets ($\times 10^9$/L)		404.1	167.7
WBC ($\times 10^9$/L)		12.6	4.8
Neutrophils ($\times 10^9$/L)		5.6	2.65
Biochemical parameters		Median	IQR
LDH (U/L)		636.3	473.4
Total bilirubin (mg/dL)		2.6	1.9
HBB cluster haplotype		n	%
Bantu/Bantu		38	54.3
Senegal/Senegal		11	15.7
Benin/Benin		3	4.3
Compound heterozygous		17	24.2
Atypical		1	1.4

A total of seventy-one genetic variants were identified of which twenty-eight (MAF > 0.05) were used in the association studies – seven in *VCAM1*, five in *NOS3*, three in *GOLGB1*, one in *PON1*, one in *ENPP1*, one in *HBA* (-$\alpha^{3.7kb}$–thal) and ten in *ITGA4* (Appendix Table A.1). We were able to reconstruct 16 haplotypes, and used ten of them (frequency > 0.05) for statistical analysis. Concerning the *HBB* gene cluster haplotypes, only the more frequent genotypes, Bantu/Bantu and Senegal/Senegal, were used for statistical analysis (frequency > 0.05).

3.2. In silico analysis

In silico analysis of the *VCAM1* gene promoter variants was focused on those with MAF > 0.05, except rs3917025 due to its occurrence in only one haplotype (Haplotype 3). The rs1041163_C, rs1409419_T and rs3917025_delCT *VCAM1* alleles were classified (according to ClinVar and Ensembl's VEP and Mat Inspector) as intergenic variants with potential modifying impact, although with no major pathologic effects. Potential changes resulting from the presence of the rs1041163_C allele include (i) TFBS alteration for RXRF transcription factor, substituting it for a PRDF site, and (ii) loss of a FHXB TFBS. The presence of rs1409419_T would lead to a potential gain of binding sites, in particular, for EVI1, Oct1 and Barx2. Regarding rs3917025_delCT, a potential gain of a FAST1 (FoxH1) TFBS was indicated.

Table 2
Association of biochemical and hematological parameters of SCA patients with stroke and stroke risk.

Parameter (units)	n	Stroke		p
		Yes	No	
		Medians (IQR)	Medians (IQR)	
HbF (%)	64	3.2 (9.3)	11.9 (10.3)	0.018
MCH (pg)	70	21.2 (20.8)	27.4 (6.0)	0.005

Parameter (units)	n	Stroke risk		p
		High + moderate	Low	
		Medians (IQR)	Medians (IQR)	
HbF (%)	64	8.5 (10.2)	12.1 (10.7)	0.043
Platelets ($\times 10^9$/L)	61	442.0 (156.4)	363.2 (124.2)	0.017
Neutrophils ($\times 10^9$/L)	61	6.3 (2.5)	4.9 (2.6)	0.009
LDH (U/L)	65	761.5 (535.7)	510.0 (325.3)	< 0.001

Values indicated as medians (interquartile range in brackets); n - number of patients; HbF - hemoglobin F; MCH - mean corpuscular hemoglobin; LDH - lactate dehydrogenase.

3.3. Association of biochemical and hematological parameters with cerebral vasculopathy

We observed significant associations of both stroke and stroke risk with several biochemical and hematological parameters (Table 2). Lower HbF percentages and MCH values were positively associated with stroke, while stroke risk was associated not only to lower HbF percentage but also to higher levels of coagulation, inflammation and hemolysis markers (Table 2).

3.4. Association of genetic variants with biochemical and hematological parameters

Genetic variants analyzed in our study, using the dominant genetic test model, showed association with both hematological and biochemical parameters, whether individually or as part of specific haplotypes (Table 3). *VCAM1* rs1041163 (CC + TC), *VCAM1* haplotype 3, *ITGA4* rs113276800 (CA) and *ITGA4* rs3770138 (TT + CT) showed an association with lower levels of HbS. Conversely, higher HbF percentages were observed in association with rs1041163 (CC + TC), *VCAM1* haplotype 3 and with Senegal/Senegal haplotype.

Namely, *VCAM1* as well as *ITGA4* variants, individually or within a haplotype context, were significantly associated with traditional biomarkers of disease severity, such as lower HbS percentage and higher LDH and total bilirubin values.

As for *PON1* rs662, the AA and GA genotypes showed a positive association with high platelets counts characteristic of a pro-coagulant state. Regarding *GOLGB1*, no significant association was found between the presence of rs3742410_C and hematological or biochemical parameters. However, we found two other *GOLGB1* SNPs while analyzing rs3732410_C – rs61746571_G and rs33988592_A, a synonymous and a missense variant, respectively. The rs61746571_G seems to be in linkage disequilibrium with variant rs3732410_C. On the other hand, rs33988592 AA and GA genotypes showed an association with lower values of inflammation markers (Table 3).

Table 3
Genetic variants association with the hematological and biochemical parameters.

Gene	Variant	Allele change or haplotype	Parameter (unit; nr. patients)		p
			HbS (%; n = 60)		
			Var	*No var*	
VCAM1	rs1041163	T > C	74.5	83.5	0.019
	Haplotype 3	C/C/CT/T/C/C	73.7	81.4	0.034
	rs33988592	G > A	11.3	13.4	0.030
ITGA4	rs113276800	C > A	69.0	80.4	0.012
	rs3770138	C > T	66.1	80.7	0.003
			HbF (%; n = 64)		
			Var	*No var*	
VCAM1	rs1041163	T > C	13.0	7.0	0.014
	Haplotype 3	C/C/CT/T/C/C	14.5	9.3	0.005
HBB	Haplotype	Sen/Sen	13.6	9.2	0.038
			Hb (g/dL; n = 65)		
			Var	*No var*	
HBB	Haplotype	Sen/Sen	8.1	7.9	0.022
			RBC ($\times 10^{12}$/L; n = 64)		
HBA	$-\alpha^{3.7kb}$ del	$\alpha\alpha > -\alpha^{3.7kb}$	3.1	2.7	0.008
			MCV (fL; n = 69)		
NOS3	rs2070744	T > C	87.3	79.9	0.024
	Haplotype IV	T/4a/G	78.7	83.2	0.032
HBA	$-\alpha^{3.7kb}$ del	$\alpha\alpha > -\alpha^{3.7kb}$	75.7	85.7	< 0.001
HBB	Haplotype	Sen/Sen	90.1	80.6	0.039
			MCH (pg; n = 70)		
			Var	*No var*	
HBA	$-\alpha^{3.7kb}$ del	$\alpha\alpha > -\alpha^{3.7kb}$	25.1	29.0	0.001
			RDW (n = 70)		
			Var	*No var*	
NOS3	rs2070744	T > C	19.6	21.6	0.031
	Haplotype I	C/4a/G	19.6	21.5	0.044
HBA	$-\alpha^{3.7kb}$ del	$\alpha\alpha > -\alpha^{3.7kb}$ del	20.8	21.2	0.021
			Platelets ($\times 10^9$/L; n = 61)		
			Var	*No var*	
PON1	rs662	G > A	442.0	378.1	0.028
			WBC ($\times 10^9$/L; n = 67)		
GOLGB1	rs33988592	G > A	11.3	13.4	0.030
HBB	Haplotype	Sen/Sen	9.9	13.0	0.030
			Neutrophils ($\times 10^9$/L; n = 61)		
			Var	*No var*	
GOLGB1	rs33988592	G > A	5.0	6.2	0.013
HBA	$-\alpha^{3.7kb}$ del	$\alpha\alpha > -\alpha^{3.7kb}$	5.7	5.5	0.010
HBB	Haplotype	Bantu/Bantu	6.2	5.0	0.036
	Haplotype	Sen/Sen	4.7	6.2	0.014
			LDH (U/L; n = 65)		
			Var	*No var*	
VCAM1	rs1409419	C > T	748.0	517.0	< 0.001
	Haplotype 7	T/C/CT/T/T/C	748.0	517.0	< 0.001
ITGA4	Haplotype A	G/GA/TT	611.5	1269.0	0.003
			Bilirubin (mg/dL; n = 69)		
			Var	*No var*	
VCAM1	rs3783613	G > C	3.2	2.4	0.026

Var – presence of variant allele or haplotype; ***No var*** – absence of variant allele or haplotype; HbS – hemoglobin S; HbF – fetal hemoglobin; Hb – total hemoglobin; RBC – red blood cells; MCV – mean corpuscular volume; MCH – mean corpuscular hemoglobin; RDW – red cell distribution width; WBC – white blood cells; LDH – lactate dehydrogenase; Sen - Senegal.

Table 4
Genetic variants association with cerebral vasculopathy.

Gene	Variant	Stroke (n = 62)				
		Yes	No	p	OR	95% CI
VCAM1	rs1409419_TT + CT	12	23	0.041	4.17	1.04–16.73
	rs1409419_CC	3	24			
	Haplotype 7	12	23	0.041	4.17	1.04–16.73
	Haplotype X	3	24			
ITGA4	rs113276800_CA	4	3	0.025	7.62	1.39–41.65
	rs113276800_CC	7	40			
	rs3770138_TT + CT	4	4	0.045	5.57	1.12–27.67
	rs3770138_CC	7	39			

Gene	Variant	SCI (n = 53)				
		Yes	No	p	OR	95% CI
NOS3	VNTR_4b	6	42	0.030	0.10	0.01–0.69
	VNTR_4a + 4c	3	2			
	Haplotype V	4	36	0.031	0.18	0.04–0.81
	Haplotype Y	5	8			

Gene	Variant	CV (n = 62)				
		Yes	No	p	OR	95% CI
NOS3	Haplotype VII	1	23	0.006	0.08	0.01–0.69
	Haplotype Z	13	25			

Gene	Variant	Stroke risk (n = 60)				
		High + moderate	Low	p	OR	95% CI
VCAM1	rs1409419_TT + CT	23	11	0.009	4.71	1.57–14.13
	rs1409419_CC	8	18			
	Haplotype 7	23	11	0.009	4.71	1.57–14.13
	Haplotype X	8	18			
NOS3	Haplotype V	18	24	0.050	0.29	0.09–0.96
	Haplotype Y	13	5			
ENPP1	rs1044498_AA + CA	14	5	0.026	4.03	1.21–13.42
	rs1044498_CC	16	23			

Haplotype X – presence of any of the other *VCAM1* promoter haplotypes studied; Haplotype Y – presence of any of the other *NOS3* haplotypes studied; SCI – silent cerebral infarction; CV – cerebral vasculopathy; 95% CI – 95% confidence interval.

3.5. Association of genetic variants with cerebral vasculopathy and cerebral vasculopathy risk

We found a significant association between the presence of several of the variants identified and CV/CV risk (Table 4). Namely, *VCAM1* rs1409419 (TT + CT), haplotype 7, *ITGA4* promoter rs113276800 (CA) and rs3770138 (TT + CT), showed a positive association with stroke. *ITGA4* variant rs3770138 (TT + CT) was also positively associated with CV as a whole. Positive associations were also found between high TAMMV values and *VCAM1* rs1409419 (TT + CT), *VCAM1* promoter haplotype 7, and also with the *ENPP1* rs1044498_A allele. On the other hand, *NOS3* intron 4 VNTR_4b allele and haplotype V were negatively associated with SCI, while haplotype VII showed a negative association with CV overall.

4. Discussion

Our study aimed to assess demographic, clinical, biochemical, hematological, genotyping and imaging data to design a potential biomarker panel for CV prognosis in children with SCA. With this approach, we were able to identify statistically significant associations of biochemical, as well as hematological parameters, with genetic variants and CV.

In silico analysis of the *VCAM1* rs1409419_T allele indicated particularly interesting potential TFBS gain, namely for EVI1, Oct1 and Barx2 transcription factors. EVI1 is of special note due to its complexity, multiple targets and modulation of several numerous processes, including cell migration, adhesion and proliferation [16]. It may cooperate with FOS transcription factor to limit cell adhesion while enhancing cell proliferation [16]. Conversely, Oct1 is a TF known to promote a transcriptional repression/silencing effect, which would potentially result in *VCAM1* down-regulation. On the other hand, a Barx2 binding site gain, predicted as a result of the rs1409419_T presence, has been shown to promote murine muscle cell differentiation, by interacting with muscle regulatory factors, whereas its loss would lead to decreased adhesion properties [17]. Hence, it is reasonable to assume that a gain could result in increased adhesion properties. All the TFs which expression may be affected by the significantly associated variants are mainly involved in development and in different tissues following the proposed role of VCAM-1 [18]. It is important to emphasize that while we can address the potential effects of the individual *VCAM1* variants' genotypes, the most important modifying role on disease manifestation would probably arise in the context of the haplotypes that encompass them. The one exception seems to be rs1409419_T given the overlapping findings observed for this variant and haplotype 7, which includes it.

We found that variants in the gene that encodes the VCAM-1 ligand, ITGA4, namely rs113276800_A and rs3770138_T were also positively associated with stroke. Furthermore, the latter was associated with CV globally. Given its role in WBC, reticulocyte and sickle erythrocyte adhesion to the activated SCA endothelium, this finding further underlines the role of cell-endothelium adhesion in the severe CV subphenotype. *ITGA4* rs113276800 has been previously described in association with multiple sclerosis and, as in our case, no AA individuals were observed [19,20]. This variant is located in the *ITGA4* promoter region near the AP-2 binding sites and the AA genotype may, therefore, cause a negative expression of the integrin α4 subunit [21]. We also found that the *ITGA4* rs1375493, rs35723031 and rs10562650 variants behaved similarly in our group of patients, most of whom were heterozygous for the three of them simultaneously. Furthermore, the co-occurrence of minor alleles in haplotype A – which, to our knowledge, has not been previously described – was associated with lower LDH values and potentially to a protective effect against hemolysis.

NOS3 (or eNOS) is the major NO-producer enzyme in the cardiovascular system, playing a crucial role in vascular tone control, vascular remodeling and proliferation. Furthermore, in SCA, NO bioavailability plays a very important modulating role, primarily through scavenging by cell-free hemoglobin [22]. The rs2070744 variant, located at position −786 of the *NOS3* gene 5′ flanking region, has been correlated with cardiovascular disease, namely its C allele, although there is still debate about how it affects mRNA and protein levels. In our study, we did not find any association of this variant with CV or stroke risk, which may be in accordance with previous reports of no significant differences between CC and TT genotypes effect on *NOS3* promoter activity [23]. The fact that the rs2070744_C has different distributions in different ethnic backgrounds [24] may also be responsible, to some extent, for these differences, since that allele is more frequent in Caucasians and our study population is of sub-Saharan origin. However, we observed a significant association between CC and TC genotypes of this variant and lower RDW, which has been discussed as a possible biomarker of lower cerebrovascular disease risk [25]. Lower RDW values (or reduced anisocytosis) would potentially act as a protective factor in consonance with what we observed for *NOS3* haplotype V, which includes allele C, and CV protection. Although rs2070744 has been described in association with cardiovascular disease [26], its role in ischemic stroke has not been consensual. *NOS3* haplotype V also includes intron 4 VNTR_4b allele, a variant that showed a protective effect against SCI. However, no relationship with any of the CV presentations studied here was observed. On the other hand, the *NOS3* rs1799983_T allele, which leads to aspartate for glutamate substitution at eNOS position 298, has been previously reported to be related to deficient eNOS caveolar localization and deficient shear stress response leading to reduced enzymatic activity. This SNP has been found in some populations to be more prevalent in patients with coronary artery disease, ischemic stroke and arterial hypertension [27]. However, in our study population, we did not observe any relationship of rs1799983 TT or GT genotypes with CV, biochemical or hematological parameters.

Several studies have identified other candidate gene polymorphisms as potentially affecting the risk of CV. However, the results published so far have been conflicting. A GWAS study by Flanagan et al. [9], performed in a large cohort of mainly African American SCA pediatric patients, showed a decreased risk of clinically overt stroke in association with *GOLGB1* rs3732410_G (Y1212C) and *ENPP1* rs1044498_C (K173Q) mutations, whereas *PON1* rs662_C (Q192R) was associated with increased risk of stroke [9]). In the same study, *GOLGB1* Y1212C was associated with reduced TCD velocities and lower frequencies of SCIs. Conversely, reports from Martella et al. [7] and Belisário et al. [10] indicated a link between *ENPP1* rs1044498_A and increased stroke risk as well as high TCD velocities. In our study, the *ENPP1* rs1044498_A allele was found in 18% of patients compared to 68.33% of Martella et al. [7], 26.08% of Belisário et al. [10] and 5.08% of Flanagan et al. [9], while homozygosity for the *GOLGB1* rs3732410_G allele was not found in our patients as in Flanagan's studies but contrary to 1.67% in Martella's report. Homozygotes for the *PON1* rs662_C allele occurred in a frequency of 10,3% in our study, whereas Martella et al. [7] and Flanagan et al. [9] reported 45% and 13.7%, respectively. Of the three variants, only *ENPP1* rs1044498 AA and AC genotypes showed a positive association with stroke risk. Notably, rs1044498_A is the minor allele, in our study group, while the variant allele (C) is the most frequent, which is in line with the reference population (African Yoruba) and contrary to what is described for the other reference populations. This may reflect a negative selection for the less advantageous allele - rs1044498_A in these populations. As for the *PON1* rs662_C variant, albeit no association with stroke or global CV was apparent, we observed a positive association with high platelet levels, indicating a potential impact on hemostasis and inflammation.

The only consensual modifiers of SCD disease severity, so far, have been the persistence of HbF beyond infancy and the presence of $-\alpha^{3.7kb}$–thal deletion. The co-inheritance of $-\alpha^{3.7kb}$–thal and homozygous HbS mutation has been associated with an overall ameliorating effect on anemia, particularly a protective effect against stroke in children [28]. We did not find any direct relationship between the presence of the $-\alpha^{3.7kb}$–thal deletion and stroke, although we did find that patients with α–thal showed a higher RBC count, lower MCV and MCH, consistent with previous reports [29,30] of ameliorating anemia factors. Other authors have also reported an absence of association between $-\alpha^{3.7kb}$–thal presence and stroke protection [31,32]. Despite the small sample size in our study, we cannot exclude that population heterogeneity or other specific population characteristics may contribute for the lack of association observed. Additionally, the unexpected association with increased neutrophil count might lower the above mentioned potentially favorable effect by indicating a proinflammatory role. The latter was also found in subjects with the Bantu haplotype while the Senegal haplotype seems to have the opposite effect, ameliorating inflammation and the hematological indices. Nevertheless, in our study, no *HBB* cluster haplotypes were found to be associated with CV.

5. Conclusion

Although the sample size in our study limits extrapolation to the general SCA pediatric population, our results seem to reinforce the importance of genetic modulators in the pathophysiology of cerebral vasculopathy and provide clues for the discovery of novel targets for SCA therapy. Our findings lead us to suggest that a comprehensive biomarker panel that includes biochemical, hematological, imaging as well as genetic data may be very important for CV prediction, and prevention. Even though the patients we studied are subjected to environmental, both physical and social, factors different from those to which the populations their parents originated from have been exposed, the genetic modifiers described in our study, namely *VCAM1* haplotype 7 and rs1409419, may provide further tools for CV prevention in SCA. Functional studies are of the utmost importance, not only for confirmation purposes, but also to assess the mechanisms by which the phenotype modulation may occur, and the potential use of these variants as novel genetic biomarkers of disease prognosis.

Author statement

Due to the sensitive nature of the question asked in this study, survey respondents were assured raw data would remain confidential and would not be shared.

CRediT authorship contribution statement

Marisa Silva: Methodology, Software, Formal analysis, Investigation, Data curation, Writing - original draft.**Sofia Vargas:**Methodology, Software, Formal analysis, Investigation,

Data curation.**Andreia Coelho**:Methodology, Software, Formal analysis, Investigation, Data curation.**Emanuel Ferreira**:Methodology, Software, Investigation.**Joana Mendonça**:Methodology, Software.**Luís Vieira**:Methodology, Software, Funding acquisition.**Raquel Maia**:Resources, Writing - review & editing. **Alexandra Dias**:Resources, Writing - review & editing.**Teresa Ferreira**:Resources, Writing - review & editing.**Anabela Morais**:Resources.**Isabel Mota Soares**:Resources, Writing - review & editing.**João Lavinha**:Funding acquisition, Writing - review & editing.**Rita Silva**:Resources, Writing - review & editing.**Paula Kjöllerström**:Resources, Writing - review & editing.**Paula Faustino**:Project administration, Funding acquisition, Methodology, Supervision, Writing - review & editing.

Declaration of competing interest

The authors have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcmd.2020.102436.

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8.2. APPENDIX B

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Hemorheological alterations in sickle cell anemia and their clinical consequences – The role of genetic modulators

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Abstract. Sickle cell anemia (SCA) is an autosomal recessive disease caused by the HBB:c.20A>T mutation that leads to hemoglobin S synthesis. The disease presents with high clinical heterogeneity characterized by chronic hemolysis, recurrent episodes of vaso-oclusion and infection. This work aimed to characterize by *in silico* studies some genetic modulators of severe hemolysis and stroke risk in children with SCA, and understand their consequences at the hemorheological level.

Association studies were performed between hemolysis biomarkers as well as the degree of cerebral vasculopathy and the inheritance of several polymorphic regions in genes related with vascular cell adhesion and vascular tonus in pediatric SCA patients. *In silico* tools (e.g. *MatInspector*) were applied to investigate the main variant consequences.

Variants in vascular adhesion molecule-1 (*VCAM1*) gene promoter and endothelial nitric oxide synthase (*NOS3*) gene were significantly associated with higher degree of hemolysis and stroke events. They potentially modify transcription factor binding sites (e.g. *VCAM1* rs1409419_T allele may lead to an EVI1 gain) or disturb the corresponding protein structure/function. Our findings emphasize the relevance of genetic variation in modulating the disease severity due to their effect on gene expression or modification of protein biological activities related with sickled erythrocyte/endothelial interactions and consequent hemorheological abnormalities.

Keywords: Sickle cell anemia, *VCAM1*, *NOS3*, genetic modulators, *in silico* analysis

1. Introduction

Sickle cell anemia (SCA; OMIM #603903) is one of the most common autosomal recessive monogenic disorders worldwide. The genetic basis of the disease is an A-to-T transversion in the 6th codon of the β-globin gene (HBB:c.20A>T), located on chromosome 11 (reviewed in [23]) which gives rise to hemoglobin S (HbS) production. The pathogenesis of SCA primarily derives from the polymerization of deoxygenated HbS in the red blood cells which, in turn, leads to the distortion of the cell and its adoption of a sickled shape, reduced deformability, increased vascular adhesion, and ultimately

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to erythroptosis and hemolysis [8, 17, 21]. The disease presents with high clinical heterogeneity characterized by chronic hemolysis, recurrent painful episodes of severe vaso-oclusion and infection.

A model of disease pathophysiology has been proposed including two clinical/biological sub-phenotypes: the hemolytic-endothelial dysfunction sub-phenotype which includes stroke, pulmonary hypertension, priapism, leg ulceration and cholelithiasis, and the viscosity-vaso-oclusion sub-phenotype with relatively high hemoglobin level, microvasculature obstruction by sickled erythrocytes, tissue damage, pain crisis, acute chest syndrome and osteonecrosis [15].

As a result of hemolysis, free hemoglobin is released into plasma. Part of the free hemoglobin quickly reacts with haptoglobin and the complex is cleared from plasma. However, due to the elevated rate of hemolysis in this disease, the excess of free circulating hemoglobin scavenges nitric oxide (NO) leading to reduced NO bioavailability. The decline in blood NO concentration leads to endothelial dysfunction, over-expression of vascular adhesion molecules and impaired vasomotor tone [16]. Since endothelial function and NO metabolism are key elements in vascular homeostasis it is reasonable to assume that molecules involved in the former, the latter and/or in both may provide clues to the pathophysiology of SCA. Vascular occlusion in SCA decreases organ perfusion, leading to tissue infarction and, together with hemolytic anemia, causes the characteristic SCA clinical complications, including intermittent painful vaso-occlusive episodes, splenic autoinfarctions and consequent increased risk of infections, acute chest syndrome, pulmonary hypertension, stroke, cumulative multi-organ damage and a shortened lifespan. Therefore, vaso-occlusion is believed to occur as a multi-step process that involves interactions between sickle erythrocytes, activated leukocytes, endothelial cells (ECs), platelets and plasma proteins [8]. Recurrent vaso-occlusion, ischemia-reperfusion with consequent vascular endothelial activation and injury induce a continuous inflammatory response in SCA individuals that is propagated by the release of high levels of inflammatory cytokines, decreased NO bioavailability and oxidative stress [8]. The ECs, activated by cytokines and low NO bioavailability, may provide the basis in specific organs, like the lung, for decreased vasodilation, blood cell adhesion and micro-thrombosis. ECs have different properties in the vascular beds of different organs. Hypothetically, those in the target organs of SCA vasculopathy may be the most dependent on NO bioactivity for normal function [1].

The phenotypic heterogeneity of this monogenic disorder has long been discussed and its multifactorial-like behaviour has prompted the hypotheses that genetic modulators other than the ones involving the β-globin cluster may come into play. Hence the designation of SCA as a single gene disorder under polygenic control. In explaining altered EC function, for instance, one of the most likely candidates is *VCAM1*, the gene encoding the vascular adhesion molecule-1 (VCAM-1), a cytokine-inducible cell surface glycoprotein present in ECs in inflammatory conditions. It mediates the adhesion of monocytes and leukocytes to the endothelium, especially in small vessels [8]. VCAM-1 is also known to lead to sickled erythrocyte adhesion to the endothelial vessel wall and therefore to vaso-occlusion [1]. Another likely SCA genetic modulator known to be involved in vascular homeostasis is the gene encoding the endothelial nitric oxide synthase eNOS, *NOS3*. Contrary to VCAM-1, eNOS is a constitutive enzyme responsible for NO endothelial production, fundamental for the vasoconstriction/vasodilation balance due to its role in smooth muscle cell relaxation [18].

In silico methods may be used to provide preliminary information on the genetic variants identified and their putative structural/functional consequences when compared with the wild-type genome sequences. Several of these research tools are available for analysing variant sequences, fundamentally relying on the type of sequence change to be analysed, and on the algorithm and databases applied by the different tools. For regulatory genomic regions, such as gene promoters, software capable of predicting which changes may lead to modified gene expression levels may provide useful information on gene regulation impairment. Analysis of putative transcription factor binding sites (TFBS) constitutes a way to achieve that end and relies on assessing the degree of similarity between a given sequence and the consensus sequence corresponding to a specific transcription factor (TF).

In the present study, *in silico* tools were applied for analysis and characterization of the structure and function of *VCAM1* variants previously associated with hemolysis severity [6] and stroke risk in SCA pediatric patients. Also, one *NOS3* gene variant associated with cardiovascular risk was also analysed [25].

2. Materials and methods

In order to evaluate possible TFBS changes that might have an influence in the regulation of *VCAM1* gene expression, the wild type sequence and three variants (SNPs rs1409419, and rs1041163 and indel rs3917025) within the gene promoter region were compared *in silico*. The nucleotide sequence of the core *VCAM1* promoter and their variants were obtained from the NCBI (http://www.ncbi.nlm.nih.gov) and ENSEMBL (http://www.ensembl.org) databases, spanning from –2180 to +101 bp relative to the main transcription starting site. The *MatInspector* (www.genomatix.de) [5] *in silico* tool was used for the TFBS analysis (Table 1), with a threshold of 0.85. The following TFs were considered in particular, due to their role in inflammation, cell proliferation and oxidative stress: nuclear factor of κ light polypeptide gene enhancer in B cells (NFkB), E-twenty-six transformation-specific (ETS) TF family, GATA-binding (GATA) TF family, TATA box binding factors, specificity protein 1 transcription factor (Sp1), octamer-binding transcription factor (Oct1), and ecotropic virus integration site 1 protein homolog (EVI1). The putative TFBS resulting from the *in silico* analysis of the SNPs were cross-referenced with the experimentally validated sites described previously by [12] and [22]. The Variant Effect Predictor (from the ENSEMBL search engine) was also used to assess potential functional consequences of the *VCAM1* promoter variants.

The putative functional consequences of the nonsynonymous SNP of *NOS3* (rs1799983) were analysed through the use of the following *in silico* tools specific for evaluating missense mutations effects on protein function: Sorting Intolerant from Tolerant (SIFT) [20], Polyphen 2 [2], Domain Mapping of Disease Mutations (DMDM) and PredictSNP [4]. The latter combines predictions of eight established prediction tools (MAPP, nsSNPAnalyzer, PANTHER, PhD-SNP, PolyPhen-1, PolyPhen-2, SIFT and SNAP) and transforms the individual confidence scores to one comparable scale of 0–100%, using the values of their observed accuracies [4].

OMIM (http://www.ncbi.nlm.nih.gov/omim), ENSEMBL (http://www.ensembl.org), dbSNP (http://www.ncbi.nlm.nih.gov/SNP) and ClinVar (http://www.ncbi.nlm.nih.gov/clinvar) databases were also used to assess SNP frequencies and disease association/pathogenicity references.

3. Results

In a previous work we have performed an association study between hemolysis biomarkers (serum LDH, total bilirubin and reticulocyte count) and the inheritance of 41 genetic variants of ten candidate genes related with vascular tonus, vascular cell adhesion, inflammation, fetal hemoglobin expression, and alpha-thalassemia, in a series of 99 pediatric SCA patients [6]. Furthermore, we have performed another association study evaluating the role of the same genetic variants in stroke risk, enrolling 66 children with SCA categorised according to their degree of cerebral vasculopathy (unpublished data). In both studies, variants in a gene related with adhesion of sickled erythrocytes to vascular endothelium (*VCAM1* rs1409419, rs3917025, and rs1041163) were associated with the SCA hemolysis severity and/or with stroke risk. On the other hand, one *NOS3* gene variant (SNP rs1799983) implicated in cardiovascular risk was also analysed [25]. Thus, potential consequences of those genetic variants were evaluated *in silico* prior to *in vitro* functional studies.

Table 1
Overview of the *in silico* prediction tools used for this study

Tool	Type	Purpose	Input	Output
MatInspector	PT	Utilizes a large library of matrix descriptions (IUPAC) for transcription factor binding sites to locate matches in DNA sequences. It assigns a quality rating to matches for filtering and selection of matches.	Nt sequence, accession number, personal db	
Variant Effect Predictor	PT	Predicts the effect of variants (SNPs, insertions, deletions, CNVs or structural variants) on genes, transcripts, and protein sequence, as well as regulatory regions.	dbSNP id.	Several scores and information according to tools/datasets used
SIFT	PT	Predicts if an AAS affects protein function.	AAS, SNP or protein accession number	Score ranges from 0 to 1; 0 = neutral, 1 = damaging
PolyPhen2	PT	Predicts the possible structural and functional impact an AAS may have on a human protein using physical and comparative considerations.	Nt/AA	Score ranges from 0 to a positive number; 0 = neutral; high positive number = damaging
Domain Database for Disease Mutations	DB/PT	Domain mapping of disease mutations (DMDM) is a database in which each disease mutation can be displayed by its gene, protein or domain location.	Gene, AA, domain, SNP accession number	
PredictSNP	PT	Consensus classifier (dataset) that combines eight of the top performing tools for the prediction of the effects of mutation on protein function.	AA	Probabilities (%) provided together with annotations extracted from PMD and the UniProt database.

PT – prediction tool; DB – database; Nt – nucleotide; AA – amino acid; AAS – amino acid substitution; PMD – Protein Mutant Database.

When the three non-coding variants of *VCAM1* were searched in ClinVar and in Variant Effect Predictor (ENSEMBL), two common features emerged: (i) to date, they are considered upstream gene variants, and (ii) their probable functional impact would be as modifiers.

The TFBS analysis performed with MatInspector (Table 2) revealed several potential effects. The input data format used was the SNP database identification number, with the analysis results corresponding to the minor frequency allele. For rs1041163, the change T>C led to a potential substitution of a retinoid X receptor heterodimer binding site (RXRF) by a positive regulatory domain I-Binding

Table 2
Results of *VCAM1* promoter variant analysis using the MatInspector software

Variant	TF change	Position	Sequence
rs1041163 (allele C)	RXRF⟶ PRDM1 ('substitution') FHXB ('loss')	–1592	gatcagaGAAAttgattca ~~gggatcAGAAaaaattga~~
rs1409419 (allele T)	EVI1; Oct1; BARX2 ('gain')*	–2021	tagaaaaTATAggcata ggCATAttaatcagt ggcatatTAATcagtaaat
rs3917025 (allele del CT)	FAST1 ('gain')	–1944	caggttgagGATTgcac

*gain of several other homeoboxes.

factor) (PRDM1), and the potential loss of a fork head homologous X (FHXB). The presence of rs1409419_T allele leads to a possible gain of several homeobox TFs, in particular, EVI1, Oct1 and homeobox protein BarH-like 2 (Barx2). Regarding rs3917025_del CT, a potential gain of a forkhead activin signal transducer-1 (FAST1) was indicated.

The DMDM analysis indicated that the nonsynonymous variant p.Glu298Asp is located in the oxygenase domain-coding region of *NOS3*. This tool is linked to the OMIM database which associates this genetic variant to susceptibility for coronary heart spasm, late-onset Alzheimer's disease and hypertension. These results were consistent with the ones obtained when assessing the ClinVar database and arise from taking into account all reports of the presence of the genetic variant in association with a given phenotype.

For the analysis of the putative functional consequences three bioinformatics tools were used: SIFT, PolyPhen 2 and PredictSNP. These tools predict the consequences of mutations that are translated into amino acid changes in the protein structure, based on algorithms and comparison with known disease variation databases. The SIFT results indicated that this variant is tolerated. The PolyPhen 2 results were consistent with the SIFT ones, with a benign classification for this variant whether considering *HumDiv* or the *HumVar* databases references. The non-pathogenic category was also attributed by the combined PredictSNP tool results (83%) which are based on analyses performed by five different tools – SIFT (71%), PolyPhen 2 (74%), PolyPhen 1 (67%), SNAP (71%) and PhDSNP (78%). Nevertheless, in this case a possible association with cardiovascular disease susceptibility was considered which is in accordance with the above mentioned databases' assessment.

4. Discussion

Although the major genetic modifiers of SCA clinical manifestations are those affecting the fetal hemoglobin expression, *VCAM1* and *NOS3* variants have also been identified as potential modulators of the disease. In this study, the results of an *in silico* analysis of *VCAM1* rs1041163, rs1409419 and rs3917025 noncoding polymorphisms, as well as *NOS3* coding SNP rs1799983, provide some clues about possible functional roles of these genetic variants in the pathophysiology of SCA.

The three *VCAM1* promoter variants mentioned above have in common the potential for affecting this gene's expression regulation. This may occur as a result of differences in TF affinity to the altered sequence as compared to the wild type sequence. In the present work, TFBS changes were indeed observed for the three polymorphic regions. Concerning the rs1041163 G>C, a RXRF by PRDM1 substitution as well as a loss of an FHXB were indicated. PRDM1 is a transcription repressor that promotes differentiation of hematopoietic B cells and secretion of pro-inflammatory cytokines

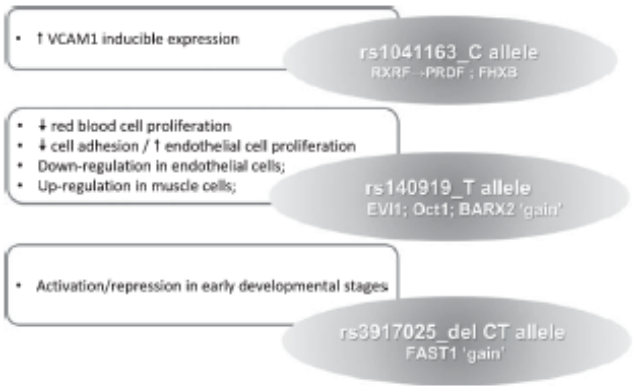

Fig. 1. *VCAM1* gene promoter variants and their predicted genetic modulation in SCA.

[9]. Therefore in a pro-inflammatory environment, such as an activated endothelium, this variant might lead to an increase of *VCAM1* inducible expression (Fig. 1). In the case of rs1409419 C>T, it was shown to have a potential gain for EVI1, Oct1 and Barx2. EVI1 is a complex multifunctional that modulates multiple processes, including cell migration, motility, adhesion, response to oxidative stress, proliferation and apoptosis/survival [3]. It contains a GATA consensus motif and prevents DNA binding by GATA1, thus limiting red blood cell differentiation and proliferation [24]. EVI1 has been reported to cooperate with FOS transcription factor to limit cell adhesion while enhancing cell proliferation, one hallmark of oncogenesis [3]. On the other hand, Oct1 is a TF known to promote a transcriptional repression/silencing effect which would potentially lead to *VCAM1* down-regulation. Barx2 has been shown to promote murine muscle cell differentiation by interacting with muscle regulatory factors (MRFs) [26], a gain of which could result in upregulation of gene expression in muscle tissue (Fig. 1). Finally, a gain of FAST1 was identified for rs3917025_delCT (Fig. 1). FAST1 is a TF involved in patterning and development of embryonic structures in vertebrates, in a complex network of activation/repression mechanisms [19].

In summary, all of the TFs affected by the sequence variants are mainly involved in development (including in early embryonic stages, as FAST1) and in different tissues which is in accordance with the VCAM-1 proposed role in development with tissue- and time-specific expression patterns [11]. In terms of endothelial environment, for instance, one might expect that altered expression levels may affect sickled erythrocytes/EC adhesion as well as endothelium inflammation/activation, thus contributing for endothelial dysfunction and ultimately to impaired blood flow/shear rate.

Regarding the rs1799983 in *NOS3*, although this SNP leads to a change in the amino acid sequence of the protein (p.Glu298Asp), all the analyses showed that this variant is most probably non-deleterious. Therefore, this *NOS3* gene variant may be considered a nonsynonymous tolerant SNP. The apparent conservative (negatively charged) amino acid substitution that results therein (aspartate for glutamate) would also be in agreement with that observation. Nevertheless, as the DMDM database results indicate, it occurs in the sequence encoding the oxygenase domain of eNOS, which is critical for the enzyme activity, containing the catalytic site as well as the components of its oxygenase function. Being considered benign, it is reasonable to assume that the variant will probably not affect the main catalytic function. Nonetheless, the oxygenase function may be impaired, thus possibly contributing to higher oxidative stress through decreased heme binding, eNOS uncoupling and (indirectly) to NO bioavailability. Possible alterations in endothelial location in the caveolae have also been proposed [14]. Overall, these potentially altered functions play key roles in endothelial dysfunction and/or vascular tone and may modulate SCA severity in terms of cardiovascular risk [13]. Furthermore, impaired oxygenase

activity would expectedly result in higher levels of oxidative stress which (i) has been demonstrated to damage healthy erythrocytes through decreasing their deformability as well as increasing the strength of erythrocytes aggregates, and (ii) have been hypothesized to induce an exaggerated response in erythrocytes from SCA patients, accompanied by a highly abnormal hemorheological profile (reviewed in [7]).

Besides its hemorheological importance, altered oxygenase function may also have an impact on therapeutic approaches. For instance, it is known that drugs interfering with the renin-angiotensin-aldosterone system, as well as statins, are useful in preventing endothelial dysfunction. However, the mechanisms through which they promote eNOS uncoupling, in the case of elevated oxidative stress, may provide useful clues as to ways of increasing NO beneficial actions in the cardiovascular system [10].

5. Conclusions

In silico studies require a careful analysis of specific factors. Concerning input data format, specific sequences already identified in databases provide lesser margin for error so, using an identification number is in general less prone to error than the manual introduction of a given sequence, for instance. The algorithm that is used for the prediction also determines the tool's accuracy, since it determines sensitivity, specificity and has associated false-positive and false-negative rates. Furthermore, the cut-off values or parameter thresholds are key elements in determining reliability of results since they are associated with the similarity of the given sequence and a reference sequence and therefore with the likelihood of a specific TF actually binding to that given sequence. Database information, as well as size, quality and curation also impact a tool's quality and reliability.

Nonetheless, *in silico* approaches only constitute a preliminary step in evaluating genetic variant potential biological and clinical consequences. *In vitro* (and, whenever possible, *in vivo* and *ex vivo*,) studies are crucial for confirmation purposes and to unravel the biological link between genetic variants and the sub-phenotypes of SCA. Gene expression studies are also of the utmost importance in particular in the case of *VCAM1* polymorphisms to assess overall up- or down-regulation of the gene, as a consequence of the changes in the regulatory sequence. In *NOS3* functional analysis is also mandatory to evaluate enzymatic activity in different cellular environments.

All studies undertaken to identify genetic modifiers of SCA sub-phenotypes are important in order to pinpoint essential pathways and mechanisms for SCA pathophysiology and to evaluate potential molecular targets to which direct innovative therapeutic strategies.

Acknowledgments

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8.3. APPENDIX C

Ph.D. Portfolio

At the different stages of this Ph.D. project, I was able to present the corresponding partial results on different platforms. The corresponding published papers, oral communications, and conference posters are listed in table 8.1.

Table 8.1. Publications presented in the course of the Ph.D. project

Type of Publication	year
Articles in Peer-Reviewed Journals	
Silva M, Vargas S, Coelho A, Ferreira E, Mendonça J, Vieira L, …, and Faustino P. (2020). *Biomarkers and genetic modulators of cerebral vasculopathy in children of African ancestry with sickle cell anemia*. Blood Cells Mol Dis, 83.102436. DOI:10.1016/j.bcmd.2020.102436	2020
Silva M, Vargas S, Coelho A, Ferreira E, Mendonça J, Vieira L, …, and Faustino P. (2020). *Moduladores genéticos de vasculopatia cerebral em crianças com drepanocitose*. Boletim Epidemiológico Observações, 9(26):63-67. http://hdl.handle.net/10400.18/7090	2020
Nicolau M, Vargas S, **Silva M**, Coelho A, Ferreira E, Mendonça J, …, and Faustino P. (2019). *Genetic modulators of fetal hemoglobin expression and ischemic stroke occurrence in African descendant children with sickle cell anemia*. Ann Hematol, 98:2673-81 DOI:10.1007/s00277-019-03783-y	2019
Ferrão J, **Silva M**, Gonçalves L, Gomes S, Loureiro P, Coelho A,…, and Faustino P. (2017). *Widening the spectrum of deletions and molecular mechanisms underlying alpha-thalassemia*. Ann Hematol, 96(11):1921-1929. DOI: 10.1007/s00277-017-3090-y	2017
Silva M, Vargas S, Coelho A, …, and Faustino P. (2016). *Hemorheological alterations in sickle cell anemia and their clinical consequences – The role of genetic modulators*. Clin Hemorheol Microcirc, 64:859-866. DOI: 10.3233/CH-168048	2016
Oral Communications	
Silva M, Vargas S, Coelho A, Ferreira E, Mendonça J, Vieira L, …, and Faustino, P. (2018). *Genetic Modulation of Cerebral Vasculopathy in Children with Sickle Cell Anemia*. January 8th, Seminars of the Departamento de Genética Humana. Instituto Nacional de Saúde Dr. Ricardo Jorge, Lisbon, Portugal	2018
Silva M, Vargas S, Coelho A, Ferreira E, Mendonça J, Vieira L, …, and Faustino P. (2017). *Endothelial factors and stroke risk in pediatric sickle cell anemia patients: insights from VCAM1 and ITGA4 variants*. 21st Annual Meeting of the Portuguese Society of Human Genetics. November 17th. Aldeia dos Capuchos, Almada, Portugal	2017

Silva M, Vargas S, Coelho A, Ferreira E, Mendonça J, Vieira L, …, and Faustino P. (2016). *Hemorheological alterations in sickle cell anemia and their clinical consequences: the role of genetic modulators*. 18th Conference of the European Society of Clinical Hemorheology and Microcirculation. June 5th. Faculty of Medicine, University of Lisbon. Lisbon. Portugal	2016

Poster Presentations

Silva M, Vargas S, Coelho A, Lavinha J, and Faustino P. (2020). *Macro- and microvascular endothelial dysfunction modulation by VCAM1 haplotypes of pediatric sickle cell anemia patients*. European Hematology Association Virtual Meeting EHA2020. June 11th -22nd.	2020
Silva M, Vargas S, Coelho A, Lavinha J, and Faustino P. (2019). *VCAM1 modulation on endothelial cells – implications for vasculopathy in sickle cell anemia*. 23rd Annual Meeting of the Portuguese Society of Human Genetics. November 14th – 16th. Coimbra, Portugal	2019
Silva M, Vargas S, Coelho A, Ferreira E, Mendonça J, Vieira L, …, and Faustino P. (2019). *Pediatric cerebral vasculopathy in sickle cell anemia – contribution of genetic modifiers.* ESH International Conference: *Erythropoiesis control and ineffective erythropoiesis: from bench to bedside*. March 15th – 17th. Budapest, Hungary	2019
Silva M. (2019). *Genetic modulation of stroke in children with sickle cell anemia*. Técnico Ph.D. Open Days. April 9th -10th. Instituto Superior Técnico, Lisbon, Portugal	2019
Ferrão J, **Silva M**, Gonçalves L, Gomes S, Loureiro P, Coelho A,…, and Faustino P. (2017). *Alpha-thalassemia due to novel deletions and complex rearrangements in the subtelomeric region of chromosome 16p*. Dia do Jovem Investigador do INSA. May 8th, Lisboa, Portugal	2017
Ferrão J, **Silva M**, Gonçalves L, Gomes S, Loureiro P, Coelho A,…, and Faustino P. (2017). *Unusual molecular mechanisms in the origin of alpha-thalassemia*. 22nd Congress of the European Hematology Association. June 22nd -25th. Madrid, Spain	2017
Ferrão J, **Silva M**, Gonçalves L, Gomes S, Loureiro P, Coelho A,…, and Faustino P. (2016). *Novel deletions and unusual genetic mechanisms underlying alpha-thalassemia*. 20th Annual Meeting of the Portuguese Society of Human Genetics. November 10th – 12th. Coimbra, Portugal	2016