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New mechanisms involved in the modulation of sickle red blood cells rheology and senescence

Sofia Esperti

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Ecole Doctorale Interdisciplinaire Science-Santé**

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New mechanisms involved in the modulation of sickle red blood cells rheology and senescence

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Abbreviations

A

ACS: acute chest syndrome

ADP: Adenosine diphosphate

AHA: acute hemolytic anemia crises

AMP: Adenosine monophosphate

AQP-1: Aquaporin-1

AQP-3: Aquaporin-3

ATG: Anti-thymocyte globulin

ATP: Adenosine-triphosphate

B

Baso-E: basophilic

C

Cat: Catalase;

CBD: Cannabidiol

CCCP: Carbonyl cyanide m-chlorophenyl hydrazone

cf-mtDNA: circulating mitochondrial-DNA

cGMP: cyclic guanosine monophosphate

CHA: chronic hemolytic anemia

CTX: Charybdotoxin

Camp: cyclic adenosine monophosphate phosphodiesterase

D

2D: 2-dimensional

2-3 DPG: 2-3 Diphosphoglycerate

DAMPs: Damage associated molecular patterns

E

Ei: Elongation index

Eimax; maximum Elongation index

Eimin: minimum Elongation index

EMA: European Medicines Agency

eNOS: endothelial NO-synthase

Epo: Erythropoietin

F

FCCP: carbonyl cyanide-p-trifluoro-methoxyphenyl-hydrazone

Fe³⁺: Iron

FSC: Forward Scatter

G

G6PD: Glucose-6-Phosphate-Dehydrogenase

GPA: Glycophorine A

GPx: Glutathione peroxidase

GR: Glutathione reductase

GSH: reduced Glutathione

GSSG: oxidized Glutathione

H

H₂CO₃: carbonic acid

H₂DCFDA: Dichlorodihydrofluorescein diacetate

H₂O₂: Hydrogen Peroxyde

HbA: Hemoglobin A

HbAC: hemoglobin C trait

HbAS: sickle cell trait

HbC: hemoglobin C

HbF: fetal hemoglobin

HbS: hemoglobin S

HbSS: sickle cell anemia homozygous individuals

HCO₃⁻: bicarbonate ion

HSP90: Heat shock protein 90

HSPCs: hematopoietic stem and progenitor cells

HU: Hydroxyurea

HX: hereditary xerocytosis

I

ICAM-1: intracellular adhesion molecule 1

ICAM-4: intracellular adhesion molecule 4

IL-1: Interleukin-1

IL-1 β : Interleukin-1 β

IL-6: Interleukin-6

IL-8: Interleukin-8

ITIMs: immunoreceptor's tyrosine-based inhibition motifs

J

JAK3: Janus kinase 3

K

KCC: K-Cl cotransport

KCC: KCl cotransporter

L

LN: Laminin

L: length

Lu/BCAM: Lutheran (Lu) blood group and Basal Cell Adhesion Molecule (BCAM)

M

MAPK: mitogen-activated protein kinase

MBE method: Macey, Bennekou and Egee method

MCHC: mean corpuscular hemoglobin concentration

MCV: mean cell volume

MFI: Mean fluorescence intensity

MP: microparticles

mtROS : mitochondrial ROS

N

N₂: Nitrogen

NADP⁺: oxidized Nicotinamide Adenine dinucleotide Phosphate

NADPH: Reduced Nicotinamide Adenine Dinucleotide Phosphate

NETs: neutrophil extracellular traps

NF-κB: Nuclear factor K-light chain enhancer of activated B cells

NIX: NIP3-like protein X

NLRP3: NOD-like receptor family, pyrin domain containing 3

NMDAR: N-Methyl-D-aspartate Receptors

NO: Nitric oxide

O

O₂: Oxygen

O₂⁻ : Superoxide Anion

OrthoE: orthochromatophilic erythroblasts

P

3-PG: 3-Phosphoglycerate

6PGD: 6-Phosphogluconate dehydrogenase

6PGL: 6-phosphogluconolactonase

PCO₂: Pressure in Carbon dioxide

PGK: Phosphoglycerate kinase

PINK1: PTEN-induced kinase 1

PO₂: Partial oxygen pressure

Poly-E: polychromatophilic

PoS: Point of Sickling

PPP: Penthose Phosphate Pathway

Pro-E: proerythroblasts

PS: Phosphatidylserine

R

r: radius

R: Relaxed

RBCs: Red Blood Cells

ROS: Reactive Oxygen Species

RT: Room temperature

S

SCA: sickle cell anemia

SCD: sickle cell disease

SLE: Systemic Lupus Erythematosus

SOD: Super Oxide Dismutase

SSC: Side Scatter

T

Δ^9 THC: Δ^9 -tetrahydrocannabinol

T: Temperature

T: Tense

TEM: transmission electron microscopy

TIA: ischemic attack

TLR4: toll-like receptor 4

TNF- α : Tumor necrosis factor α

TRPV2: transient receptor potential vanilloid 2

TRPV2-KO: TRPV2-Knock Out

U

US FDA: United States Food and Drug Administration

V

VOC: vaso-occlusive crisis

VCAM-1: Vascular cell adhesion protein 1

VDAC1: protein voltage-gated anion channel 1

VSMCs: vascular smooth muscle cells

W

WHO: World Health Organization

WT: wild type

X

XO: xanthine oxidase

Summary and Context of the Thesis

This thesis was part of a collaborative project called “**EVIDENCE**”, a Research Fellowship Program between 15 different laboratories in Europe, all highly specialized in the study of red blood Cells properties and/or their progenitors, their maturation, and red blood Cell diseases.

Integrating the research group « LIBM - Laboratoire Interuniversitaire de Biologie de la Motricité (UR7424) » in Lyon, I mainly worked in the Vascular Biology and Red Blood Cell team, on red blood cells characterization, especially in sickle cell disease.

Sickle cell disease is the most common inherited blood disorder in the world. Even if the “discovery” of the disease has been done a long time ago (early 1900), lot of research works are still being performed to understand pathophysiological mechanisms and new factors which could exacerbate the outcome of the disease, and to propose new therapies for the patients.

Indeed, even if this genetic condition is caused by a single point mutation in the genome, its pathophysiology is characterized by complex mechanisms which can lead to various clinical profiles or complications. Through the characterization of red blood cells from patients with sickle cell disease and the analysis of new parameters, we propose new factors which could impact the red blood cells properties, such as their rheological characteristics and senescence.

1. Red blood cells characteristics

The Red Blood Cells (RBCs), also called erythrocytes, represent the most abundant cell type in the human body, characterized by a shaped-like biconcave disk with an average diameter of around 6 to 8 μm . The periphery of the disk is thicker than the flattened middle part and the biconcave shape gives RBCs a larger surface area, a feature that facilitates the gas exchange inward and outward the RBC (Figure 1). The biconcave shape is determined by the presence of a complex cytoskeleton consisting of filaments binding transmembrane proteins, which allows RBCs to be highly deformable and pass-through blood capillaries¹.

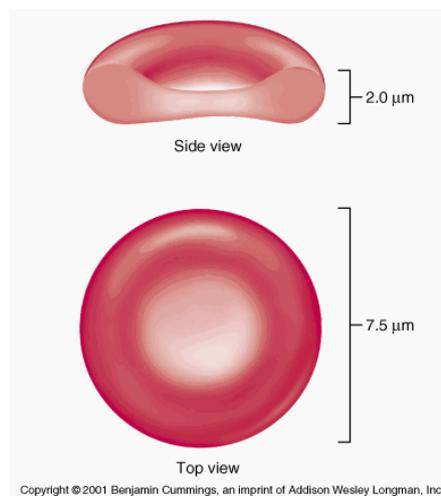


Figure 1. Shape and size of red blood cells. From https://www.google.com/search?q=red+blood+cell+size&rlz=1C1CHBD_frIE924IE924&sxsrf=AJOqlzVF_a_cUV62oC3ShcC0lwH3JMe5AJA:1676295135996&source=lnms&tbm=isch&sa=X&ved=2ahUKEwimjfqAzpL9AhUMVaQEhUCxAZMQ_AUoAXoECAEQAw&biw=1280&bih=577&pr=1.5#imgsrc=FSJIUmK0EUIvhM.

RBCs lack nucleus and organelles in contrast to many other cells type of the human body. The lifespan of RBCs in the bloodstream is approximately 120 days, then RBCs are removed from the circulation through a cellular process called phagocytosis,

which takes place mainly in macrophages of the spleen, but also in the liver or the bone marrow. The most important function of RBCs is the delivery of oxygen from the lungs to tissues and carbon dioxide from tissues to the lungs². Only 1.5% of oxygen can dissolve in blood, most oxygen (95%) diffuses from the alveoli of the lungs into RBCs, where it binds to a metalloprotein, the hemoglobin. In healthy adults, the predominant form of hemoglobin is hemoglobin A, a globular protein made of four polypeptide chains, two alpha chains, and two beta chains. Each chain has a heme-group containing Iron (Fe^{2+}), which has a high affinity for oxygen (O_2) and can bind one molecule of O_2 . Thus, each hemoglobin molecule can bind four molecules of O_2 . Hemoglobin exists in one of two conformations, called T (tense) and R (relaxed)³. Deoxygenated hemoglobin has the T conformation, but the binding of oxygen molecules triggers the change to the R state. Due to its allosteric properties, the binding of the first oxygen molecule increases the affinity of hemoglobin for oxygen and promotes the fixation of the other oxygen molecules.

Several environmental factors modulate the affinity of hemoglobin for O_2 ⁴. The most important is the association between partial pressure in O_2 (PO_2) and hemoglobin oxygen saturation, which describes the oxyhemoglobin dissociation curve. The curve is described by a sigmoid plot because of the cooperative binding of O_2 with the 4 polypeptide chains.

In the lungs, at high PO_2 , hemoglobin binds to oxygen and forms oxyhemoglobin. When the blood is fully saturated with oxygen, all the hemoglobin is in the form of oxyhemoglobin. At low PO_2 , as in the tissues, oxyhemoglobin releases oxygen to form

deoxyhemoglobin. However, the PO_2 in the tissues is never too low to allow a complete desaturation of the four O_2 molecules from the hemoglobin, and this feature allows the hemoglobin to have a “stock” of O_2 in case of high physical or environmental stress³.

Several factors can increase or decrease the affinity of hemoglobin for oxygen, such as the pH, the partial pressure in carbon dioxide (PCO_2), the temperature (T), and the 2,3-diphosphoglycerate (2,3-DPG) concentration (a metabolite produced during anaerobic glycolysis). The increase of PCO_2 , 2,3-DPG, temperature, and low pH decrease the affinity of hemoglobin for oxygen, causing the shift of the curve to the right. The decrease of PCO_2 , 2,3-DPG, temperature, and the increase of pH increase the affinity of hemoglobin for oxygen, causing the shift of the curve to the left (Figure 2).

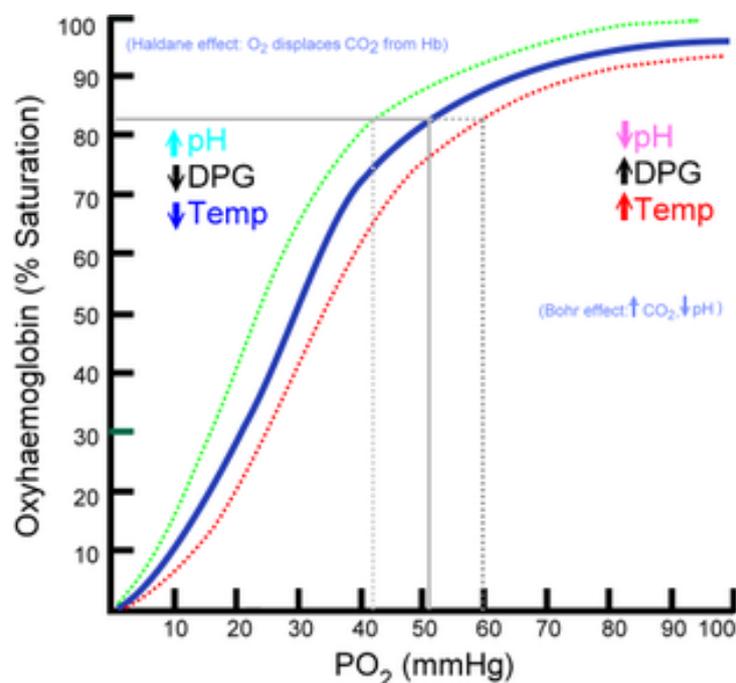


Figure 2. Oxyhemoglobin dissociation curve. The shift to the left (green line) or to the right (red line) of the curve, which means the increase or decrease in the affinity of hemoglobin for O₂, is influenced by the increase or decrease of several factors: 2,3-diphosphoglycerate (2,3-DPG), pH, temperature (T). From https://en.wikipedia.org/wiki/Oxygen%E2%80%93hemoglobin_dissociation_curve.

Respiratory gases move across membranes by simple diffusion, following their partial pressure gradient. Therefore, CO₂ will tend to move from a compartment with a high PCO₂, to another one with a lower PCO₂. According to the same principle, O₂ will move from the capillaries (where PO₂ is higher) to the cells where PO₂ is lower since O₂ is continuously consumed by mitochondria (cellular respiration) and reduced to water⁵.

Most of the CO₂ is transported into the plasma in the form of the bicarbonate ion (HCO₃⁻), while O₂ travels bound to hemoglobin within red blood cells. When CO₂ enters the bloodstream, the enzyme carbonic anhydrase quickly converts it into carbonic acid (H₂CO₃), which dissociates into H⁺ ions and bicarbonate ions (HCO₃⁻) (Figure 3). The formation of H⁺ ions causes the decrease of the pH (which becomes more acidic), promoting the detachment of O₂ from hemoglobin to the tissues: this is the Bohr effect⁶.

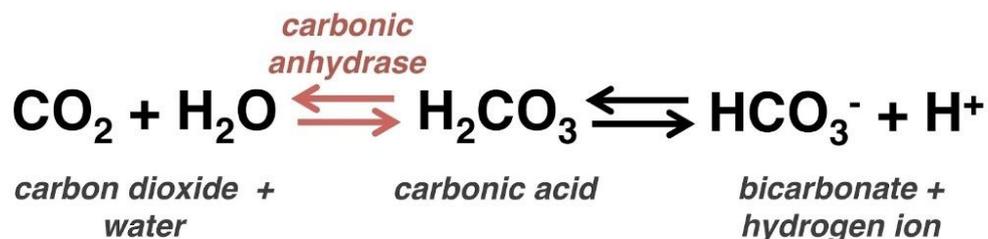


Figure 3. Hydration of carbon dioxide into carbonic acid and dissociation into H⁺ and bicarbonate ions. From <https://www.toppr.com/ask/question/carbonic-anhydrase-is-playing-an-important-role-in-transport-of-carbon-dioxide-in/>.

Similarly, in the alveoli lungs, there is much O_2 and low CO_2 because of inspiration. The O_2 diffuses into RBCs binding the hemoglobin and reducing the hemoglobin affinity for CO_2 and protons. The effect of O_2 on the binding of protons and CO_2 on the hemoglobin is called the *Haldane effect*. Then, protons react with bicarbonate ions creating carbonic acid, which is transformed into CO_2 and water. Then, the CO_2 diffuses in the alveoli, and it can be exhaled⁷. (Figure 4).

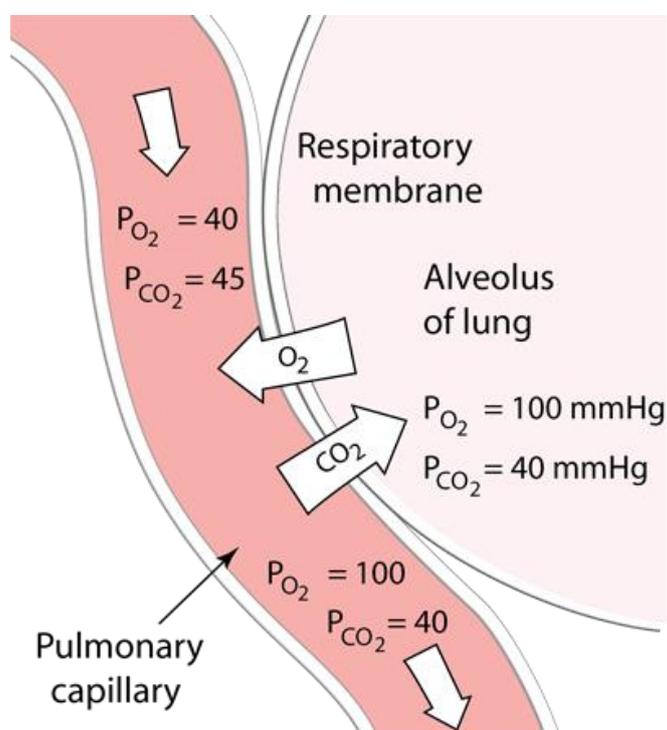


Figure 4. Transport of gases between the environment and alveoli. Image from <http://hyperphysics.phy-astr.gsu.edu/hbase/Organic/hemo.html>.

1.1 Types of Hemoglobin

Several types of hemoglobin exist α , β , γ and δ Hemoglobin which differ from each other only for a few amino acids. As previously described, the four globin chains in

the human hemoglobin from adults (HbA) are made of two α -chains and two β -chains. HbA percentage in normal adults is about 97% of the total hemoglobin. Only a small percentage which comprises the remaining 3% of adult hemoglobin, is represented by another HbA form (HbA₂), composed of two α chains and two δ chains. Another type of hemoglobin is present in the fetus during gestation, called fetal hemoglobin (HbF). HbF is composed of two γ chains and two α chains. It delivers oxygen from maternal to fetal circulation, so it has a greater oxygen affinity than HbA. About six months after birth, in the newly synthesized RBCs, HbF is replaced by HbA⁸ (Figure 5).

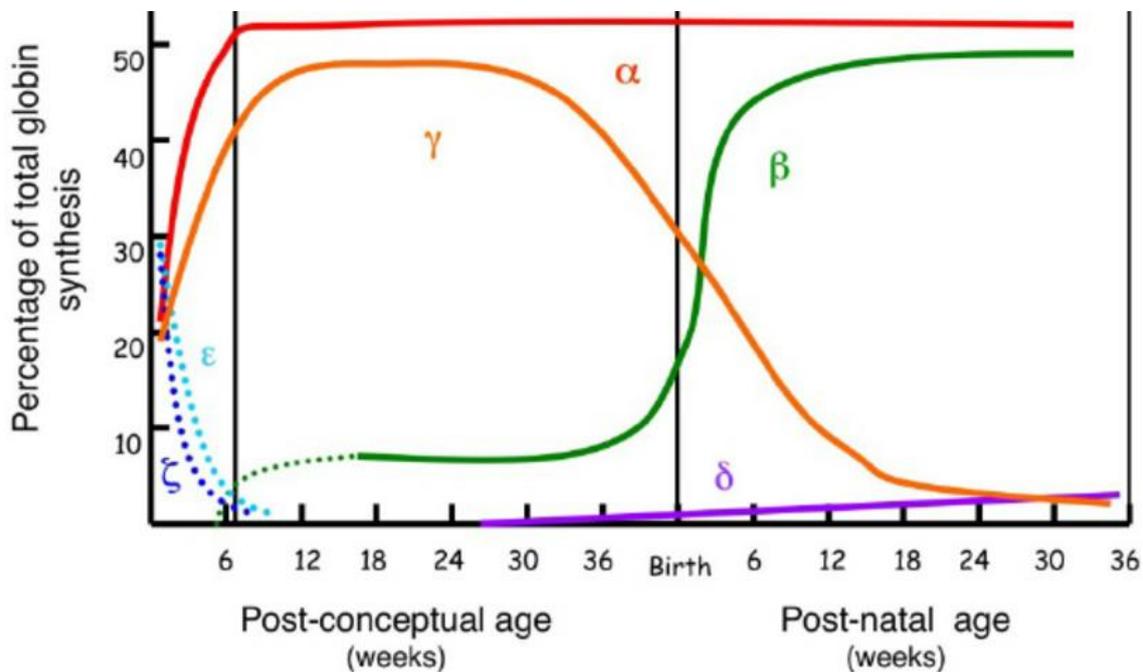


Figure 5. Gene expression before and after birth. Hemoglobin γ is replaced by Hemoglobin β after birth. From Grosso et al, 2012⁹.

1.2 Red Blood Cell metabolism

RBCs lack nuclei and organelles, and they can't synthesize new proteins or renew the components of their cellular membranes. Indeed, RBCs only have an anaerobic metabolism to provide Adenosine triphosphate (ATP)¹⁰.

1.2.1 Glycolysis and anaerobic metabolism of red blood cells

The lack of a nucleus and mitochondria do not allow RBCs to generate energy via the Krebs cycle, which represents an oxidative pathway that uses O₂ (cellular respiration) to produce energy. Anaerobic oxidation of glucose, also known as the Embden–Meyerhof pathway, is the only energy source for RBCs. Under normal physiological conditions, most glucose is catabolized anaerobically to pyruvate or lactate, and during this degradation, two molecules of ATP are consumed, and four molecules of ATP are synthesized. This pathway ensures the maintenance of several cellular functions, such as the maintenance of the electrolyte gradient between RBCs and the extracellular environment through the activity of ATP-driven membrane pumps. In addition, the metabolic pathway is involved in the synthesis of glutathione, the maintenance of reduced hemoglobin's Fe²⁺, and the preservation of the membrane phospholipid asymmetry¹⁰ for instance. Moreover, RBCs have another, and unique metabolic pathway called the Rapoport-Luebering shunt, which is a glycolytic bypass reaction required to produce 2,3-DPG. The 2,3-DPG molecule interacts with deoxygenated hemoglobin and promotes the release of the remaining O₂ molecules bound to hemoglobin. The Rapoport-Luebering pathway bypasses the

phosphoglycerate kinase (PGK) step of the glycolysis, and accounts for the synthesis and regulation of 2,3-DPG levels which in turn is required to regulate the O₂ release from hemoglobin and delivery to tissues. Then, the diphosphoglycerate phosphatase enzyme transforms 2,3-DPG into 3-Phosphoglycerate (3-PG), which can be reused by glycolysis¹¹ (Figure 6).

1.2.2 The Pentose Phosphate Pathway

The pentose phosphate pathway (PPP), also known as the hexose monophosphate shunt, is a metabolic pathway parallel to glycolysis that characterizes RBCs, and which allows the conversion of glucose-6-phosphate to 6-phosphogluconolactone by Glucose-6-Phosphate Dehydrogenase (G6PD) enzyme, and 6-phosphogluconate to ribulose-5-phosphate by 6-phosphogluconate dehydrogenase (PGD, 6PGD). These two steps of the *PPP* are needed to reduce NADP⁺ to NADPH, which is essential in the RBCs for the regulation of oxidative stress. Indeed, NADPH is required to reduce the oxidized Glutathione (GSSG) into reduced Glutathione (GSH) (Figure 6). Glutathione is one of the most important antioxidants in RBCs; the PPP ensures the maintenance of the cellular pool of reduced Glutathione, which plays a fundamental role in the antioxidant defense of RBCs¹².

amounts of oxygen. Several reactive oxygen species (ROS) are produced in RBCs. ROS are a wide variety of unstable molecules characterized by oxygen containing highly reactive unpaired electrons, which can easily react with other molecules, causing damages. The term ROS includes superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^{\cdot}) and singlet oxygen (1O_2). These ROS can cause membrane damages to RBCs through proteins and lipids oxidation¹⁴. The ferrous iron (Fe^{2+}) of hemoglobin is exposed continuously to high concentrations of oxygen, undergoing slow oxidation to methemoglobin (containing ferric iron Fe^{3+}), forming the superoxide ions ($O_2^{\cdot-}$) which are rapidly dismutated by superoxide dismutase (SOD) enzymes to form hydrogen peroxide (H_2O_2)¹⁵. In physiological conditions, the redox state is maintained in RBCs by several antioxidant systems, which include nonenzymatic antioxidants such as reduced Glutathione (GSH), Vitamin C, and Vitamin E, and enzymatic antioxidants including glutathione peroxidase, SOD, catalase and peroxiredoxin 2¹⁶. Increased production of oxidant molecules and/or the decreased production of antioxidant agents results in an imbalance and leads to oxidative stress¹⁴.

Within RBCs, GSH is one of the most important antioxidant molecules, and its concentration in healthy RBCs is about tenfold the concentration of oxidized Glutathione (GSSG) (3 mM compared to 0.3 mM)¹⁷. GSH levels decrease in senescent RBCs in vivo, with a progressive accumulation of GSSG¹⁸. GSSG in RBCs is rapidly reduced to GSH by Glutathione reductase, an enzyme that uses NADPH as a reducing agent. In turn, the cytosolic NADPH pool is maintained in RBCs via the pentose

phosphate pathway by the G6PD enzyme. The lack of G6PD leads to an X-linked disorder called G6PD deficiency which increases the sensitivity of RBCs for oxidative stress¹⁹. Inherited deficiencies of G6PD can result in acute hemolytic anemia during times of increased ROS production. This may be caused by stress or exposure to certain foods that contain high amounts of oxidative substances, such as fava beans, or certain medications, such as anti-malarial agents²⁰.

1.4 The Red Blood Cell membrane

1.4.1 Composition

Mature RBCs are subjected to large elastic deformations which allow them to flow in the circulation and to squeeze through small capillaries during their life. Their deformability depends on several factors, but one of the most important is the nature and the properties of the RBC membrane, which is characterized by a two-dimensional structure (2D) composed of a cytoskeleton and a lipid bilayer made of a phospholipids, sphingolipids, cholesterol, and integral membrane proteins. The cytoskeleton is made up of Spectrin tetramers formed by $\alpha\beta$ -Spectrin heterodimers, and it is linked to the RBC membrane via Band-3 proteins at the Spectrin-Ankyrin binding sites, and glycophorin at the actin junctional complexes²¹. The Band-3 and the 4.1R complex are the most important complexes with a structural function in the RBC membrane and they allow the interaction between the cytoskeleton and the

membrane (Figure 7). The structure and the features of the RBC membrane are altered in some RBC membrane disorders, where a genetic mutation causes a lack in one of the key membrane proteins²². For instance, in hereditary spherocytosis, the molecular defect may involve Band-3, Ankyrin, Spectrin, or 4.1R complexes, and make the RBCs less deformable, with a spheroid shape, and more fragile²³.

RBC membrane fluidity is modulated by several factors, such as the amount of cholesterol relative to phospholipids, the type of cholesterol, and the degree of saturation of fatty acids²⁴. Despite the distribution of cholesterol being homogenous in the bilayers of the membrane, the phospholipids are distributed asymmetrically. In physiological conditions, the phosphatidylcholine and sphingomyelin are more present in the outer leaflet of the membrane, while the phosphatidylethanolamine and the phosphatidylserine (PS) are more distributed in the inner leaflet. This asymmetric but physiological distribution is maintained by several ATP-dependent enzymes: the flippases, which are responsible to transport the phospholipids from the outer to the inner side of the membrane, and the floppases, which transport the phospholipids from the inner to the outer side of the membrane²¹. In contrast, scramblases are ATP-independent enzymes which act as bidirectionally transporters of lipids between the membrane leaflets, maintaining the plasma membrane asymmetry²⁵.

The role of flippases and floppases is very important in the maintenance of PS in the inner side of the membrane. When the PS switches on the outer side, it is recognized by macrophages which can in turn phagocyte the RBC²⁶. These mechanisms which

leads to PS exposure have been largely described during the senescent stage of RBCs, but increased percentages of RBCs exposing PS are also a hallmark of some RBC diseases such as sickle cell disease²⁷.

Transmembrane proteins are another essential component of the RBC membrane. Between them, one of the most important is the Glycophorin A (GPA), also called CD235a, a transmembrane single-pass glycoprotein containing sialic acid which confers a negative charge to the RBC membrane. This feature creates a repulsive electric potential and avoid RBCs aggregation. CD235a is a marker of mature RBCs, and its expression can change during the senescence of RBCs in case of blood diseases such as sickle cell disease²⁸.

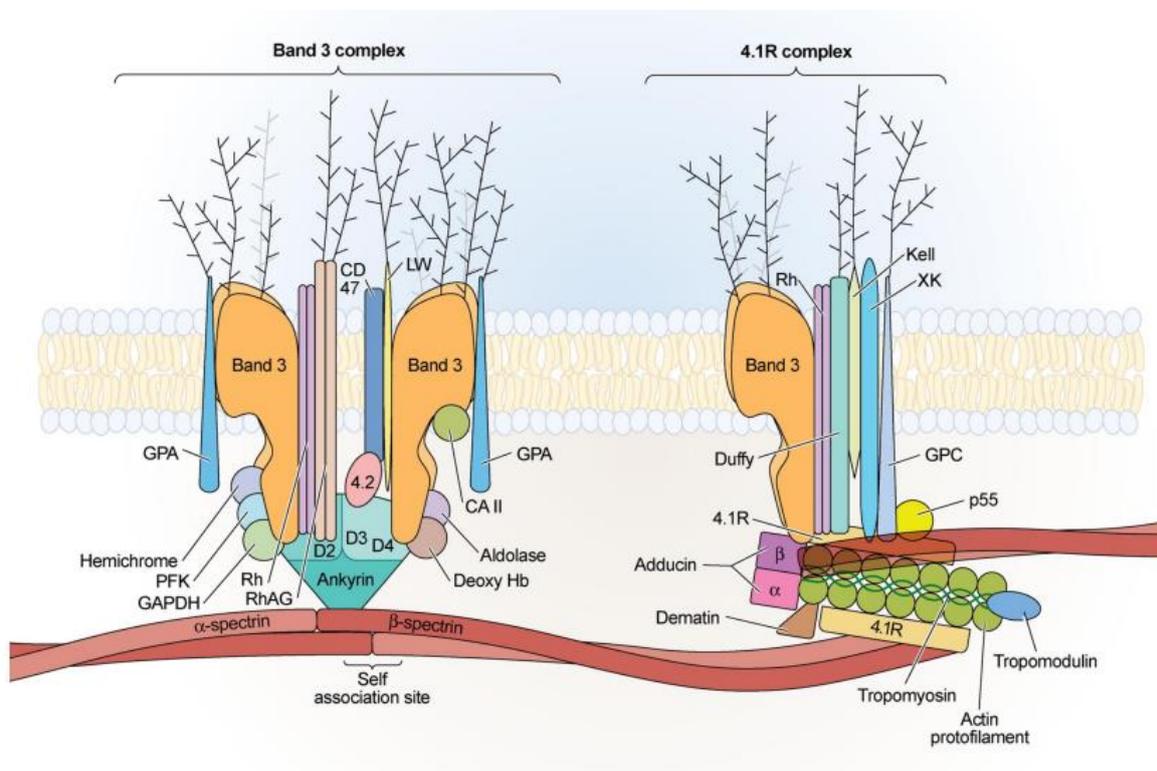


Figure 7. Structure of the red blood cell membrane. Left (Band 3 complex): Band 3 is bound to Ankyrin, which is bound to β -spectrin. Transmembrane glycoprotein GPA binds to Band 3. Right (4.1R Complex): spectrin-protein 4.1R-junctional complex linkages, and interactions between skeletal proteins and proteins of the inner component of the lipid bilayer. (From Salomao *et al.* 2008²⁹).

1.4.2 Ion Channels

The transport and exchange of respiratory gases through blood are highly dependent on the acid-base equilibrium of RBCs³⁰.

The RBC membrane contains many proteins which are responsible for ion homeostasis and water transport in and out of the cell³¹. These transmembrane proteins are called ion channels and they actively regulate ions flux and establish concentration gradients across the cell membrane. Ion channels are directly implicated in the membrane potential changes and in the regulation of cell volume, and they ensure the deformability of red cells in narrow vessels³².

Red blood cell deformability is influenced by RBC volume and ion content, both regulated by ion pumps, ion channels, symporters and antiporters³³. When ion channels are open, ions move following their electrochemical gradient, while ion pumps can actively move the ions against their gradient³⁴. The electrochemical gradient across the cell membrane is maintained by the Na⁺, K⁺ pump, which is responsible for the transport of Na⁺ outside and K⁺ ions inside the cell, resulting in volume and water homeostasis³⁵.

Thus, RBC volume changes result from different osmotic pressures, which are followed by water transport by aquaporins; in particular Aquaporin-1 (AQP1)³⁶ and Aquaporin-3 (AQP3)³⁷ which are found in human red blood cells. However, the cotransport of water is also done by the K^+ - Cl^- cotransporter KCC¹³.

Among the others ion channels on the red cell membrane, we must mention Piezo1 and Gardos channels. Piezo1 is a mechanosensitive cation channel, which means that it is activated when RBCs are subjected to mechanical forces and stretches, such as in the small vessels of microcirculation³⁸. The activation of Piezo1 leads to Ca^{2+} influx, which in turn may activate a Ca^{2+} -sensitive K^+ channel, called KCNN4 channel or the Gardos channel, from the name of the scientist who first reported the effect of Ca^{2+} on K^+ permeability³⁹. The activation of Gardos leads to an efflux of K^+ , Cl^- , and H_2O , causing dehydration and a reduction of the RBC volume. This transient and rapid mechanism facilitates RBCs to squeeze through small capillaries in the microcirculation¹³. Such an interplay between Piezo1 and Gardos channel has been demonstrated using Charybdotoxin (CTX), a small protein present in the venom of the scorpion *Leiurus quinquetriatus* or using the Gardos channel blocker TRAM34. Indeed, in the presence of CTX or TRAM34, the Ca^{2+} -activated K^+ efflux is rapidly blocked, and the hyperpolarization of the cell is inhibited⁴⁰ (Figure 8).

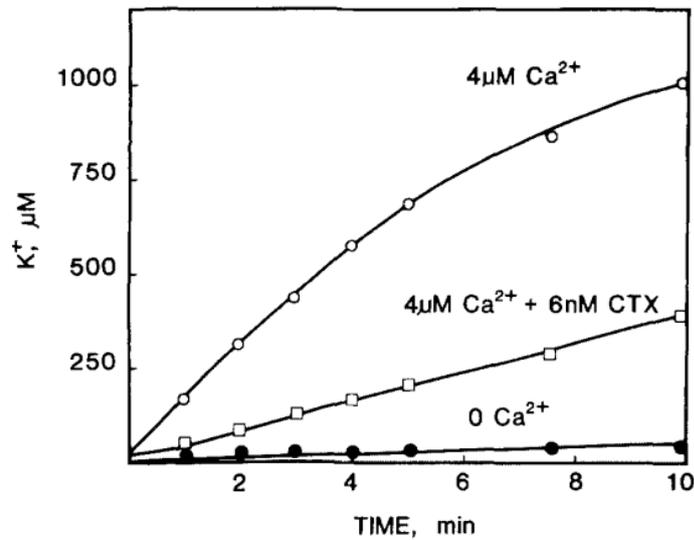


Figure 8. Measurements of extracellular K^+ concentration of RBCs resuspended in cellular medium containing Ca^{2+} and Ca^{2+} + CTX. K^+ efflux decreases using CTX. From Wolff et al, 1988⁴⁰.

The function of ion channels is involved in the change of ions distribution in and out of the RBCs. The ionic composition of the cytosol in RBCs is usually very different from that of the extracellular milieu. In normal conditions, the cytosolic K^+ concentration is about ten times greater than the Na^+ , while the opposite is observed in the extracellular environment⁴¹. This Na^+ and K^+ gradient is maintained by Na^+/K^+ -ATPase ion pumps, ensuring the activity of secondary transporters present in the membrane, such as the Gardos channel⁴².

The activation of the Gardos channel leads to the efflux of positive charges (K^+) which makes the intracellular membrane environment more negative. This process generates a voltage gradient and leads to the hyperpolarization of the membrane potential, which changes from approximately -12 millivolts (Mv) to -60Mv⁴³.

The Gardos channel number per RBC is approximately 100-200 copies per cell⁴⁴, and its channel opening is stochastic, leading the membrane potential changing with an effect called “pseudo action potential”³². The K⁺, Cl⁻, and water efflux is not the only effect of the Gardos channel activation. Indeed, the resulting hyperpolarization may induce the opening of other voltage activated Ca²⁺ channels in RBCs, such as CaV2.1 channel⁴⁵ which was reported to be abundant in RBCs⁴⁶. This mechanism could explain the higher intracellular Ca²⁺ levels in RBCs from patients having a gain of function of the Gardos channel^{47,48}.

These ion channels are essential for the survival of RBCs, and the alteration of their activity has been reported in several RBC diseases, such as hereditary Xerocytosis (HX). HX is an inherited RBCs disorder caused by the gain of function mutations in Piezo1 channel⁴⁹, resulting in dehydrated erythrocytes with a deficit in intracellular potassium, incompletely compensated by intracellular sodium. A gain of function mutation in Piezo1 has been also reported in about 20% of patients with Sickle cell disease of African origin⁵⁰, but the effect of the Piezo1 mutation on the severity of sickle cell disease is still unknown⁵¹ (Figure 9).

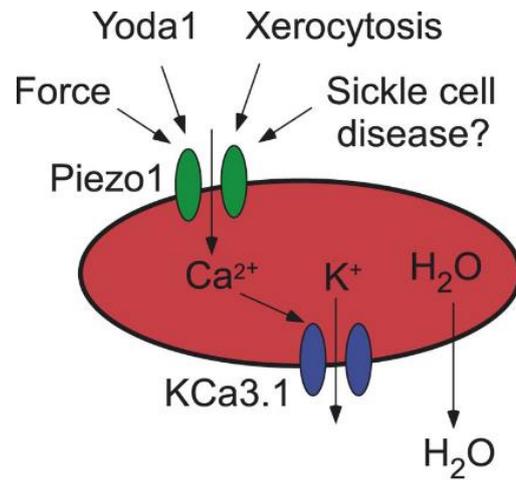


Figure 9. Piezo1 and Gardos (Kca3.1) channels on the red blood cell membrane. Activation of Piezo1 by mechanical force or chemicals (Yoda1) leads to Ca²⁺ influx and Gardos activation, followed by K⁺, Cl⁻ and water loss. Mutations of Piezo1 are reported in hereditary Xerocytosis. From Patel et al, 2015⁵².

Recently, another non-selective cation channel mainly regulating Ca²⁺ influx has been discovered in RBCs, the so-called “transient receptor potential vanilloid 2” or TRPV2 channel⁵³. TRPV2 is a nonselective cation channel that is part of the TRP subfamily and can be activated by Δ⁹-tetrahydrocannabinol (Δ⁹THC), Cannabidiol (CBD), mechanical forces or heating (57°C). TRPV2 promotes Ca²⁺ influx and consequent cell volume regulation in human and mouse RBCs, showing similar properties to those of Piezo1⁵³ (Figure 10). Indeed, in TRPV2-Knock Out (TRPV2-KO) mouse model, RBCs showed increased osmotic fragility suggesting that the TRPV2 activation in wild type (WT) RBCs causes Ca²⁺ entry and Gardos channel activation, with K⁺ and water efflux, as observed for Piezo1.

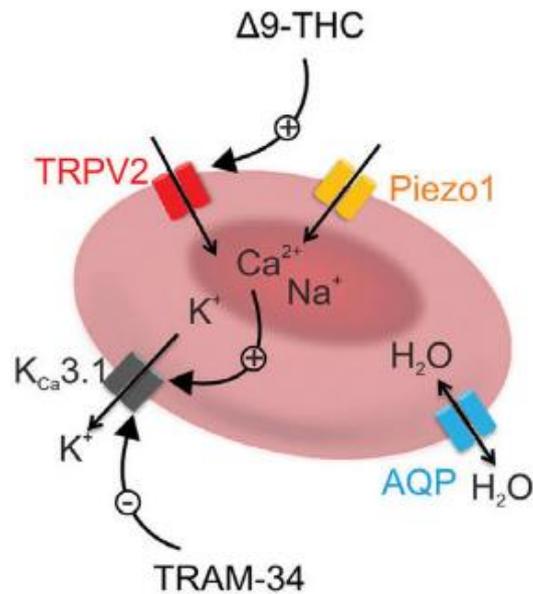


Figure 10. TRPV2 activation on RBC membrane by THC molecule. The activation of TRPV2 channel mediates Ca^{2+} influx, as observed for Piezo1, leading to K^+ loss and dehydration. From Belkacemi et al, 2021⁵⁴.

1.5 Red blood cells enzymes

1.5.1 Generalities

The maturation process of erythroblasts in RBCs involves the disappearance of almost all the metabolic pathways belonging to any other nucleated cell, leaving only the components essential for the maintenance of energy supply and for the defense against oxidative stress. Despite this limited metabolic machinery, mature RBCs are adapted to fully perform their functions of binding and delivery of oxygen during their lifespan of around 120 days in circulation. As previously introduced, RBCs are characterized by three metabolic pathways¹³:

- The Anaerobic glycolysis, which metabolizes 90% of glucose, is the only source of energy (to produce ATP) to maintain the cell structure and its function.
- The remaining amount of glucose is metabolized through the pentose phosphate pathway which is required to maintain the redox potential of NADPH. NADPH is needed as a cofactor for the glutathione reductase, which maintains reduced Glutathione levels against oxidative stress (GSH).
- Purine and pyrimidine metabolism, which is required to maintain the nucleotide cellular pool Adenosine monophosphate (AMP), Adenosine diphosphate (ADP), and ATP (Figure 11).

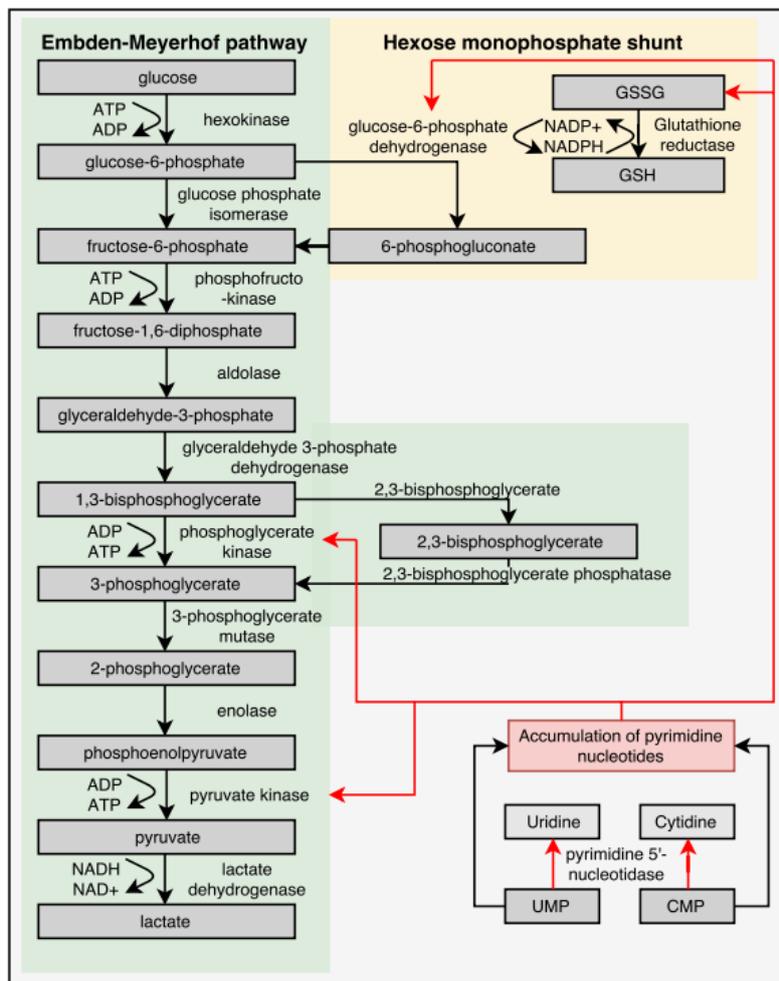


Figure 11. Glycolytic pathway and purine-pyrimidine metabolism of RBCs. The purine-pyrimidine pathway in RBCs is needed to replenish adenosine phosphate from the adenosine phosphate pool¹³.

When one or more of these metabolic pathways are defective, we talk about RBC enzymopathies, which are almost all characterized by chronic hemolytic anemia⁵⁵. In case of abnormalities of the pentose phosphate pathways and glutathione metabolism, the outcome is an acute hemolytic crisis after exposure to oxidant substances⁵⁶. The degree of hemolysis depends on the severity of enzyme deficiency, a consequence of the properties of the mutant enzyme, regarding its functional abnormalities, molecular instability, or both⁵⁷.

In addition to hemolytic anemia (chronic or acute), RBC enzymopathies are associated with non-hematological manifestations, such as neuropathy, muscle disease, infections, and metabolic acidosis⁵⁸.

Most of the RBC enzymopathies are hereditary but acquired deficiencies have also been described mainly in malignant hematological disorders. In general, the mode of inheritance is autosomal recessive for almost all RBC enzymopathies, but in the case of G6PD deficiency, it is a recessive X-linked inheritance⁵⁹.

1.5.2 G6PD deficiency

G6PD deficiency is the most common human enzymopathy, and it is estimated to affect 400 million people worldwide⁶⁰. G6PD deficiency is an X-linked disease caused by mutations in the G6PD gene located at the distal end of the long arm of the X chromosome, resulting in an inefficient expression of the gene and leading to G6PD enzyme deficiency. The expression of the genetic mutation is more common in males because the disease is X-linked; however, heterozygous females are a very common genotype⁵⁹.

G6PD catalyzes the first step in hexose monophosphate shunt, which is necessary for producing NADPH, which in turn is required for the maintenance of reduced Glutathione (GSH) (Figure 12). Because RBCs carry oxygen, they are particularly vulnerable to oxidative stress. GSH is a tripeptide that protects RBCs from oxidative

damage and is distributed in all cells. G6PD-deficient cells undergo hemolysis in response to free radicals and reactive oxygen species. G6PD deficiency may lead to acute hemolytic anemia after exposure to oxidant agents, drugs, or fava beans (favism)⁶¹.

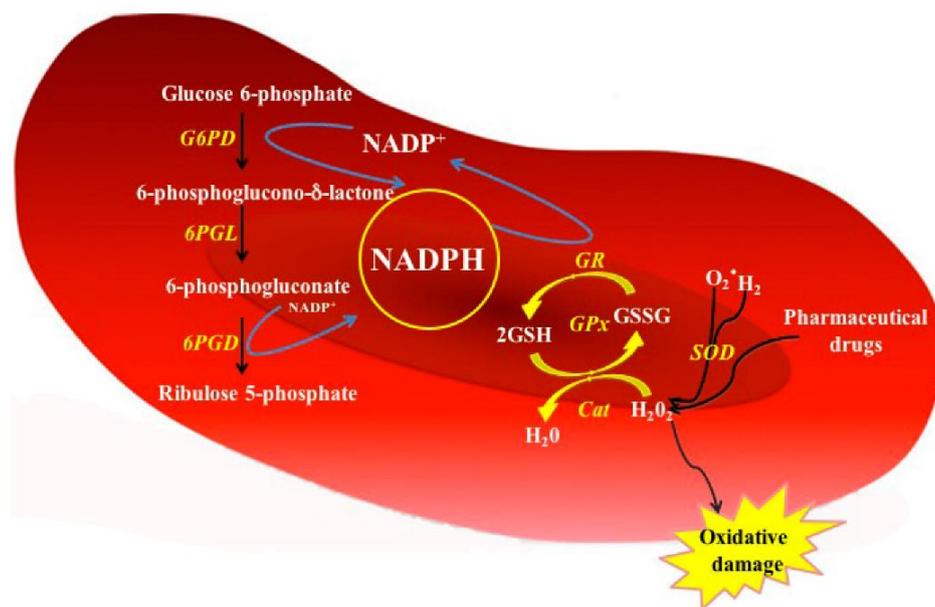


Figure 12. Pentose phosphate pathway and G6PD generation. G6PD catalyzes the formation of NADPH which is needed to maintain GSH levels high enough to fight against oxidative damage. Cat = Catalase; GPx = Glutathione peroxidase; GR = Glutathione reductase; G6PD = glucose 6-phosphate dehydrogenase; 6PGL = 6-phosphogluconolactonase; 6PGD = 6-phosphogluconate dehydrogenase; SOD = Superoxide dismutase; GSH = Reduced glutathione; GSSG = Oxidized glutathione; H₂O₂ = Hydrogen Peroxide; O₂⁻ = Superoxide Anion. From Gomez-Manzo et al. 2016⁶².

Several G6PD variants have been identified at a genetic and biochemical level, using the standard procedure for their characterization, such as enzyme activity screening, Km for G6P, heat stability, and electrophoretic pattern⁶³. A classification introduced

by the WHO (World Health Organization) has described 188 G6PD allele variants divided into five classes according to the severity of the clinical phenotype:

- Class I: severe enzyme deficiency (less than 10% of normal) with chronic nonspherocytic hemolytic anemia (CHA).
- Class II: called “Mediterranean type”, is characterized by a severe enzyme deficiency (less than 10% of normal) with acute hemolytic anemia crises (AHA).
- Class III: moderate to a mild enzyme deficiency (10% to 60% of normal) associated with AHA.
- Class IV: mild or no enzyme deficiency (60%-100% of normal).
- Class V: hyperactive enzyme activity more than twice normal.

The highest frequencies of G6PD deficiency have a global distribution, that is very similar to that of malaria (Africa, the Mediterranean area, the Middle East, and sub-tropical Asia)⁶⁴. However, because of migration, the disease is also present in North and South America, and Europe. G6PD mutations may afford protection against malaria leading support to the so-called malaria protection hypothesis, but the link between the two diseases has not been established⁶⁵.

Patients with chronic hemolytic anemia due to G6PD deficiency have in general a history of severe neonatal jaundice, blood transfusion requirement, splenomegaly, and gallstones at an early age⁶⁶. Chronic hemolysis due to G6PD deficiency may be exacerbated by the co-inheritance of other genetic RBC disorders, such as sickle cell disease⁶⁷.

Drugs or medications that can induce an acute hemolytic crisis in individuals with G6PD deficiency fall into two categories. First, there are drugs capable of causing significant hemolytic anemia even at standard therapeutic doses. Moreover, there are drugs known to affect RBC survival when administered in high doses⁶⁸.

1.6 Erythropoiesis and mitochondria clearance

Erythropoiesis is the process that produces mature red blood cells from multipotential hematopoietic stem cells⁶⁹. During mammalian development, erythropoiesis occurs successively in the yolk sac, fetal liver⁷⁰, and bone marrow⁷¹. This cellular process is finely regulated by the combined effects of microenvironmental and growth factors and is characterized by several differentiation steps which restrict the multipotent features of the cells and allows the erythroid-specific program of gene expression⁷². The production of RBCs is the largest quantitative output of the hematopoietic system, and the production rate is estimated to be around 2×10^{11} erythrocytes per day⁷³.

The first stage of erythroid maturation starts from CD34+ hematopoietic stem and progenitor cells (HSPCs) in the bone marrow within the erythroblastic islands, which consist of a central macrophage that extends cytoplasmic protrusions to surrounding erythroblasts⁷⁴ and give rise to erythroid-committed progenitors. At this stage, proerythroblasts (Pro-E) undergo morphological changes reducing cell size, starting

chromatin condensation, and synthesizing hemoglobin, reducing their proliferative capacity to give rise to basophilic (Baso-E), polychromatophilic (Poly-E) and orthochromatic (OrthoE) erythroblasts. Erythropoietin (Epo) is the main regulator of erythropoiesis, driving proliferation and differentiation and preventing apoptosis of erythroid precursor⁷⁵.

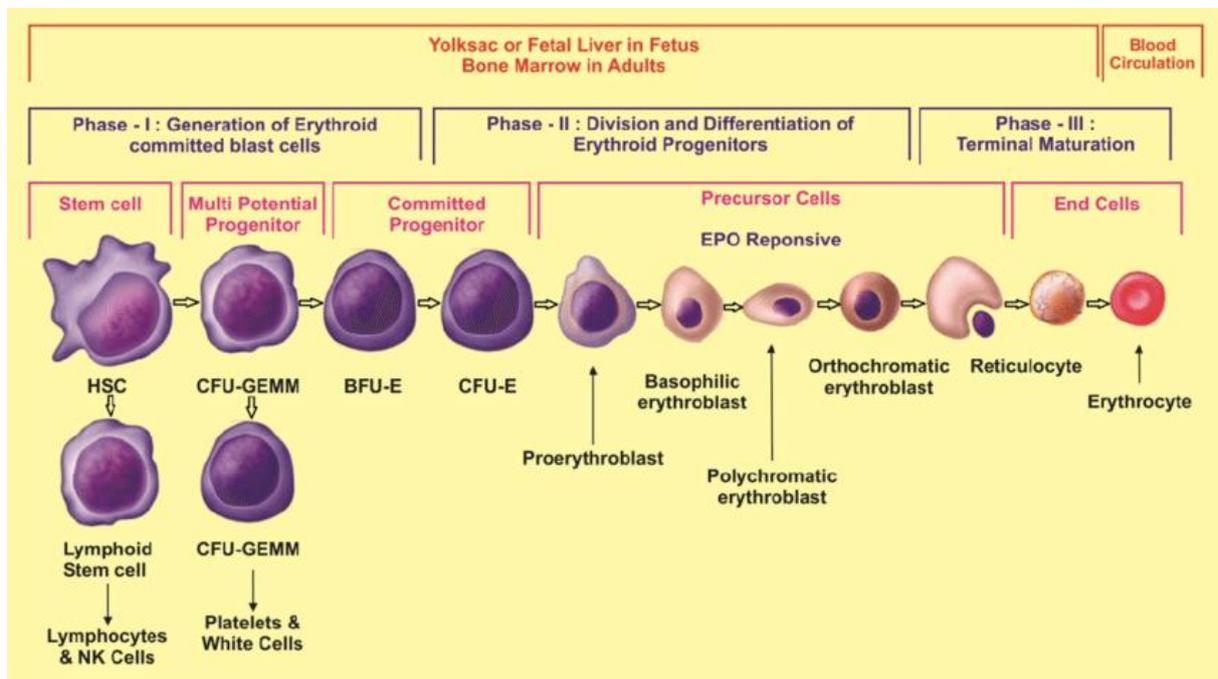


Figure 13. Schematic diagram representing the *in vivo* erythropoiesis. Differentiation of hematopoietic stem cells into red blood cells. BFU-E: blast colony forming unit-erythroid; CFU-E: colony forming unit-erythroid; CFU-GEMM: colony forming unit that generates myeloid cells; HSCs: hematopoietic stem cells⁷⁵.

Another important role during erythropoiesis is played by the mitochondria of the erythroid progenitor, which are reprogrammed to promote the differentiation process and to ensure nuclear clearance⁷⁶. Then, at the end of terminal maturation, mammalian orthochromatic erythroblasts eliminate their nuclei and organelles,

including mitochondria, shifting their metabolism to anaerobic glycolysis⁷⁷. In general, so in eukaryotic cells, mitochondria are primarily implicated in cellular bioenergetics and the regulation of critical cellular processes. The elimination of mitochondria during erythropoiesis is ensured by a process called mitophagy, a selective type of autophagy that characterizes the latest step of erythroid maturation (Figure 14).

In general, autophagy is characterized by the formation of a double membrane vesicle called autophagosome, which engulfs the organelles and delivers them to the lysosomal compartment⁷⁸. The mitophagy pathway in terminal erythropoiesis involves a specific group of proteins, which ensure the elimination of mitochondria with low membrane potential⁷⁹. Among these proteins, the mitochondrial receptor Nip3-like X (Nix) protein has been reported to be upregulated during the terminal erythropoiesis in human and mouse model. Nix is a pro-apoptotic member of the Bcl-2 family and ensures the autophagosome formation for mitochondria elimination⁷⁹. Nix KO mice showed anemia and reticulocytosis because mitochondria retention leads to ROS accumulation and hemolysis⁸⁰. Another important protein in the mitophagy process is the PTEN-induced kinase 1 (PINK1), which acts as a “quality control” for the degradation of damaged mitochondria. Abnormal mitochondria are recognized by PINK1 which binds on the outer membrane of the mitochondria and recruits parkin protein. The PINK1/parkin combination then designates the mitochondria for degradation by lysosomes⁸¹.

Mitophagy is a crucial process for RBC survival in blood circulation because mitochondria are the main sources of ROS that are produced during oxidative

phosphorylation. The accumulation of ROS may cause damage to cellular components and oxidize membrane proteins and lipids, leading to cell death⁸².

The mitochondria are emerging as highly influential organelles in the health and disease of eukaryotic cells. Mitochondrial dysfunctions or their impaired functionality are recognized as a characteristic of various diseases⁸³. Primary mitochondrial diseases are characterized by increased ROS production in mitochondria that impairs their function and dynamics, causing a continuous vicious cycle that aggravates the pathological phenotype⁸⁴.

In the last years, increased mitochondria retention within RBCs has been reported in several diseases, such as the Rett Syndrome⁸⁵, Systemic Lupus Erythematosus (SLE)⁸⁶, and sickle cell disease^{87,88}. As previously discussed, mitochondrial clearance is a feature of RBC maturation in physiological conditions. Normal healthy RBCs lack nuclei and organelles to fulfill their role as oxygen carriers and to avoid the accumulation of intracellular ROS. The abnormal retention of mitochondria within RBCs could increase oxidative stress and oxygen consumption, reducing the amount of oxygen carried through the circulation and damaging RBCs.

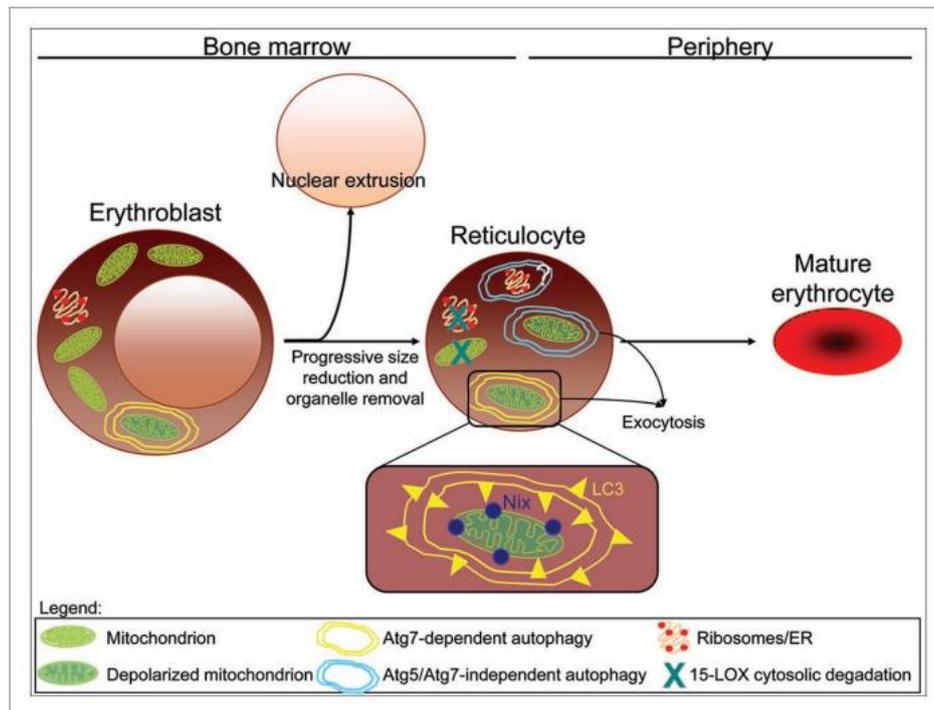


Figure 14. RBC maturation and mitochondria clearance. Mitochondria are eliminated throughout erythroid maturation, from the erythroblast until the mature erythrocyte stage. From Mortensen et al, 2010⁷⁷.

1.7 Mitochondrial respiratory chain and free radical generation

Mammalian cells generate their ATP by a process called oxidative phosphorylation, which takes place in the mitochondrial inner membrane⁸⁹. This process is carried out by two functional entities: first, the respiratory chain defined as four complexes (I, II, III and IV) that transfer electrons to molecular oxygen via electron carriers such as quinone and cytochrome C; second, the system that phosphorylates ADP to produce ATP, which comprises the ATP synthase.

The Complex I of the electron transport chain is named as NADH dehydrogenase complex, it is a very large protein complex, and it transports electrons from NADH to

Co-enzyme Q (also called Ubiquinone), a small hydrophobic molecule dissolved in the inner mitochondrial membrane which acts as an electron shuttle from Complex I and II to Complex III. The Complex I also act as a proton pump that uses the movement of electrons to move four protons to the inter membrane space. The protein Complex II, also called succinate reductase, contains the succinate dehydrogenase enzyme used in the citric acid cycle to transform succinate into fumarate, and in the process forms FADH_2 . Unlike the other protein Complexes, the Complex II isn't a proton pump and it doesn't move hydrogen ions across the inner membrane. Complex III accepts new electrons from reduced Co-enzyme Q and transports them to Cytochrome C, it acts as a proton pump and helps generate a proton electrochemical gradient. The Complex IV, also known as Cytochrome C oxidase, uses the electrons to pump H^+ ions out of the matrix, it transfers electrons onto Oxygen, reducing it to form water. Thus, the Complexes I, III and IV establish a proton gradient which is used by the ATP synthase to generate ATP molecules in the oxidative phosphorylation⁸⁹.

During the process of oxidative phosphorylation, mitochondrial ROS are generated during the electron transport chain in the inner mitochondrial membrane⁹⁰. Indeed, under physiological conditions, about the 0.2-2% of the electrons in the electron transport chain don't follow the normal transfer order, but instead directly leak out of the electron transport chain and interact with oxygen, producing ROS⁹¹.

Complexes I, II and III are thought to be major sites where superoxide anion and other ROS are produced⁹² (Figure 15). Indeed, the electrons leak from Complex I, II and III causes the O_2 reduction and the following production of superoxide anion $\text{O}_2^{\bullet-}$.

As significant ROS producers, mitochondrial structures are exposed to high ROS concentrations and may therefore be particularly susceptible to their harmful power. High oxidative stress can damage the mitochondrial respiratory chain, alter the cell membrane permeability and influence intracellular Ca^{2+} homeostasis, triggering cell death⁹³.

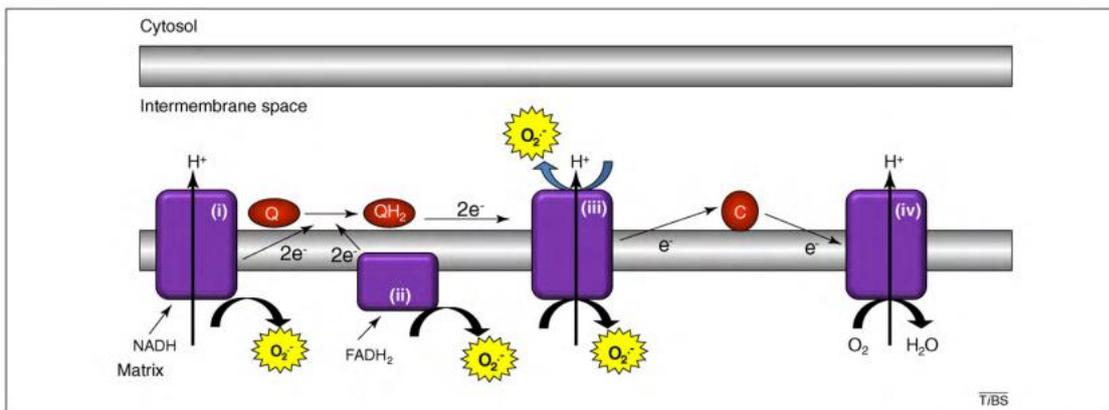


Figure 15. ROS production in the mitochondrial electron transport chain. Superoxide anion ($\text{O}_2^{\bullet-}$) is released in the mitochondrial matrix by Complex I and II. Complex III produces superoxide anion into both sides, the matrix and the intermembrane space. From Zhang et al, 2017⁹⁴.

2. Red blood cell senescence

In healthy individuals, the lifespan of circulating RBCs can vary between 100 and 120 days, at the end of which RBCs undergo senescence and are removed from circulation. During their senescence, RBCs undergo morphological and molecular alterations such as: cellular volume reduction, density and shape change, and membrane alterations⁹⁵.

Indeed, despite the absence of a nucleus and mitochondria in mature RBCs, the senescence pathway of RBCs has several similarities with apoptosis, the programmed

cell death of nucleated cells⁹⁶. During their natural aging and before their senescence, RBCs can experience injuries that can compromise their structure, function, and survival²⁶. RBC senescence is defined by a regulated process that allows the elimination of aging and damaged RBCs, avoiding their lysis and the release of hemoglobin and heme in the blood circulation⁹⁷. The induction of a senescent state involves the increased permeability of the RBC membrane for extracellular Ca^{2+} , leading to Ca^{2+} accumulation into the cells and consequent activation of caspases and calpains, the induction of PS exposure on the outer membrane of the cell, cell blebbing, and cell shrinkage⁹⁸. Early senescence of RBCs can be induced by xenobiotics, molecules inducing high oxidative stress, or in cases of energy depletion⁹⁹. Furthermore, the regulation and the alteration of RBC senescence are altered in several diseases, such as: sickle cell disease and thalassemia, G6PD deficiency, diabetes, kidney failure¹⁰⁰.

2.1 Mechanism

As previously mentioned, the increased RBC permeability for Ca^{2+} is one of the main steps of RBCs senescence which can induce cell dehydration, membrane phospholipid asymmetry alteration, translocation of the PS from the inner leaflet to the outlet one and shedding of microvesicles¹⁰¹.

The PS exposure is the result of the inhibition of flippase and the activation of scramblase, which are sensitive to increased Ca^{2+} concentrations (Figure 16). This

mechanism leads to the breakdown of the cell membrane asymmetry and makes senescent RBCs recognizable by macrophages, which will eliminate them from the blood circulation like apoptotic nucleated cells¹⁰².

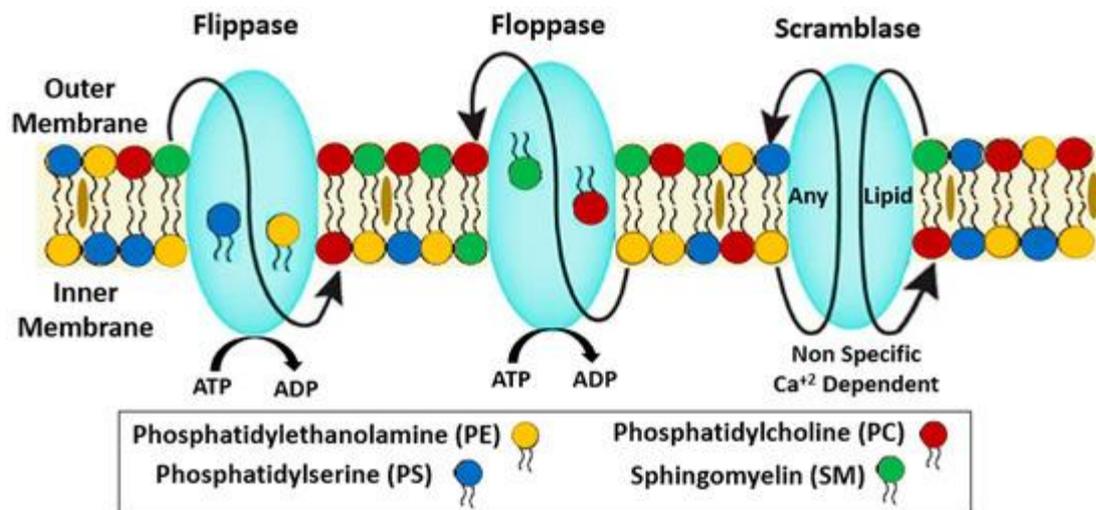


Figure 16. Representation of the lipid bilayer and enzymes involved in the maintenance of plasma membrane asymmetry. From Alghareeb et al, 2023¹⁰³.

The increase of Ca²⁺ influx depends on the activation of specific membrane proteins, called “non-selective cation channel” (Figure 17). Intracellular Ca²⁺ in turn activates the Ca²⁺ sensitive potassium (K⁺) channel, called Gardos channel, which in turn causes the K⁺ efflux and membrane hyperpolarization⁴³, followed by Cl⁻ and water loss, dehydration, and reduction of the cell volume.

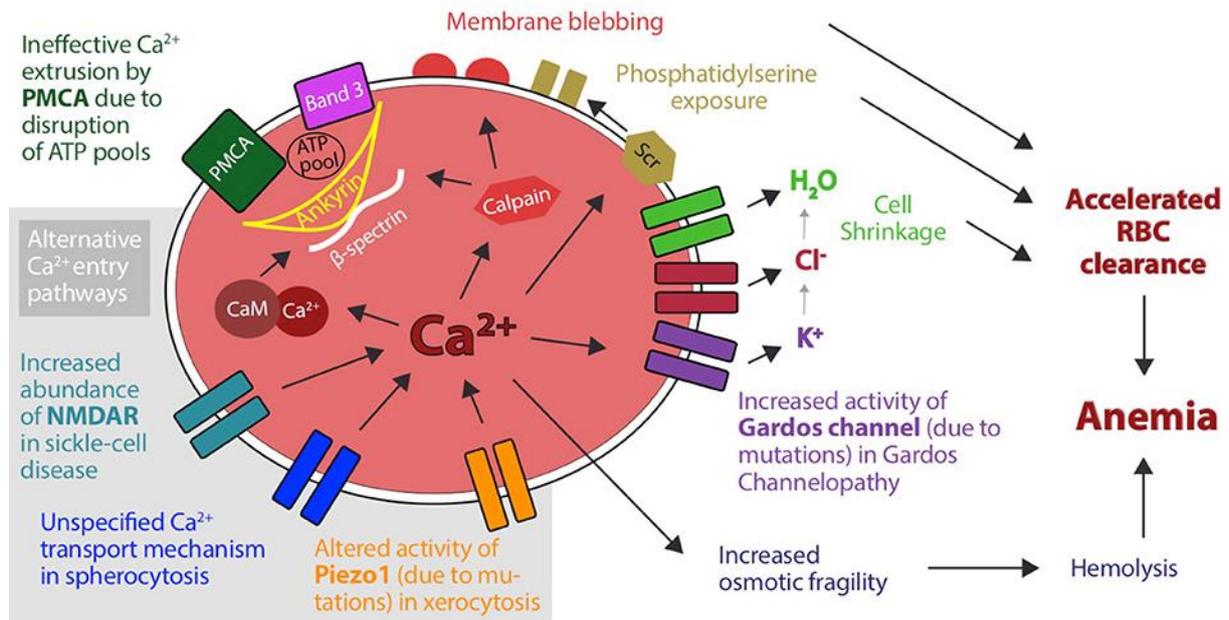


Figure 17. Mechanisms involved in increased intracellular Ca^{2+} in RBC diseases. Accumulation of Ca^{2+} accelerates RBC clearance and increases osmotic fragility, which in turn leads to hemolysis and anemia. Increased abundance of NMDA-receptors (NMDAR) in sickle cell disease; altered activity of Piezo1 in xerocytosis; increased activity of Gardos channel in Gardos channelopathy; unspecified Ca^{2+} transport mechanism in spherocytosis. Increased intracellular Ca^{2+} activates several pathways: activation of calmodulin and calpain and alteration of cytoskeleton, phosphatidylserine exposure, Gardos channel activation and cell shrinkage. From Hertz et al., 2017¹⁰⁴.

Several protein kinases are involved in the RBC senescence process, such as the p38 mitogen-activated protein kinase (MAPK) and Janus kinase (JAK3), which undergoes phosphorylation when activated⁹⁹. It has been reported that the pharmacological inhibition of MAPK and JAK3 causes the reduction of Ca^{2+} influx and PS exposure caused by osmotic shock¹⁰⁵. Furthermore, the decreased p38 MAPK activity after the addition of Ca^{2+} ionophore (ionomycin) was correlated to a reduction of the PS exposure, suggesting a role of p38 MAPK in the stimulation of RBC senescence by participating in the triggering of Ca^{2+} influx into RBCs after an osmotic shock¹⁰⁵. As in apoptotic nucleated cells, and also in senescent RBCs, the presence of protein

Caspases 3 has been reported to have a role in the PS exposure caused by oxidative stress, in the cleavage of the human Erythroid anion exchanger (Band 3) and in the uncoupling of the membrane bilayer with the spectrin-based cytoskeleton, causing the disruption of the membrane architecture¹⁰⁶.

Among all the factors that regulate the elimination of senescent RBCs, the anti-phagocytic protein CD47 plays a crucial role. CD47 is a glycoprotein that is also called “don’t eat me signal”, because its expression on the RBC surface displays a “marker of self” and inhibits the erythrophagocytosis by macrophages¹⁰⁷. CD47 binds the SIRP α receptors at the surface of macrophages, inducing inhibitory signaling by the immunoreceptor’s tyrosine-based inhibition motifs (ITIMs) on the cytoplasmic tail of the SIRP α (Figure 18). When RBCs become older, the expression of CD47 on their surface decreases as well as its inhibitory effect, and RBCs are phagocytosed by macrophages. The anti-phagocytic role of CD47 has been also reported in the mouse model, where CD47-knock-out mice had hemolytic anemia and splenomegaly¹⁰⁸.

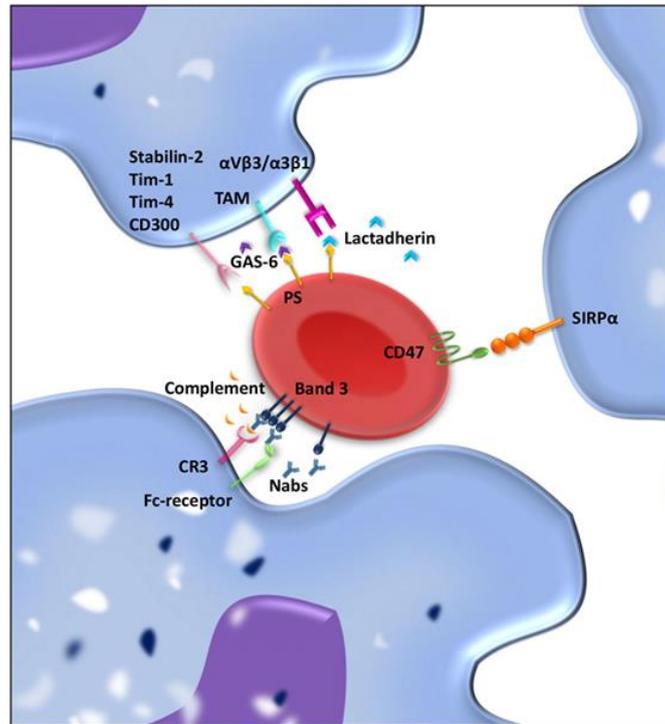


Figure 18. Promoters and inhibitors of the Interaction between mature RBC and spleen macrophages. Phosphatidylserine (PS) binding and Band 3 clustering promotes a pro-phagocytic signal, while CD47-SIRP α interactions inhibit phagocytosis. From Klei et al., 2017¹⁰⁹.

RBC aging process impairs the cellular ability to neutralize ROS, making RBCs more susceptible to oxidative damage. Accumulation of oxidative stress has several consequences on the RBC functions: it impairs oxygen delivery and causes the oxidation of proteins and lipids on the membrane, impacting the RBCs structure. Oxidized hemoglobin precipitates for hemichromes, interacting with the cytoplasmic domains of Band 3 proteins leading to reticulations and clustering of Band 3 at the RBC's surface¹¹⁰. This mechanism leads to the Band 3 dissociation from ankyrin, spectrin, and 4.1R protein, causing the disruption of the structure of the cytoskeleton and membrane shedding¹¹¹.

2.2 Phagocytosis of senescent red blood cells

Senescent RBCs are removed from the circulation by resident macrophages of the spleen, liver, and bone marrow through a process called Erythrophagocytosis. RBCs exposing senescence markers are recognized and phagocytosed, and their cellular components are “recycled” for the synthesis of new RBCs¹¹².

As previously mentioned, senescent RBCs are removed from the circulation by the resident macrophages of the spleen. The spleen is divided into two main parts: the white and the red pulp (Figure 19). The white pulp is part of the immune system because it produces lymphocytes which in turn produce antibodies. The red pulp function is to filter the arterial blood, and it is also a storage site for iron after the destruction of aged RBCs¹¹³.

The arterial blood reaches the Billroth cords which are composed of fibroblasts, reticular fibers, and macrophages. From the cords, the blood flows in the splenic sinusoids where it is filtered, and the senescent RBCs are recognized and phagocytosed by macrophages¹¹⁴.

Aged RBCs show several changes, such as decreased cell volume, morphological alterations, and decreased metabolic activity. Some molecular alterations occur at the surface of RBCs, like PS exposure and decreased expression of CD47, and promote the interaction between aged RBCs and macrophages. Besides, RBCs with reduced deformability are unable to squeeze through endothelial slits in the human

spleen. Thus, they are retained in the splenic chords and destroyed by red pulp macrophages. The main determinants of the red cell deformability are the ratio of cell surface area to volume intracellular viscosity (determined by the properties of hemoglobin) and membrane elasticity (which depends on the rheological properties of the membrane⁹⁷. The loss of red blood cell deformability is due to the cell dehydration and shrinking, and to the loss of cell membrane surface triggered by the membrane blebbing and microparticles production¹⁰¹. The loss of deformability decrease the ability of red cell to flow and facilitates the remotion of senescent and less deformable RBCs by macrophages in the narrow vessels of the spleen⁹⁷.

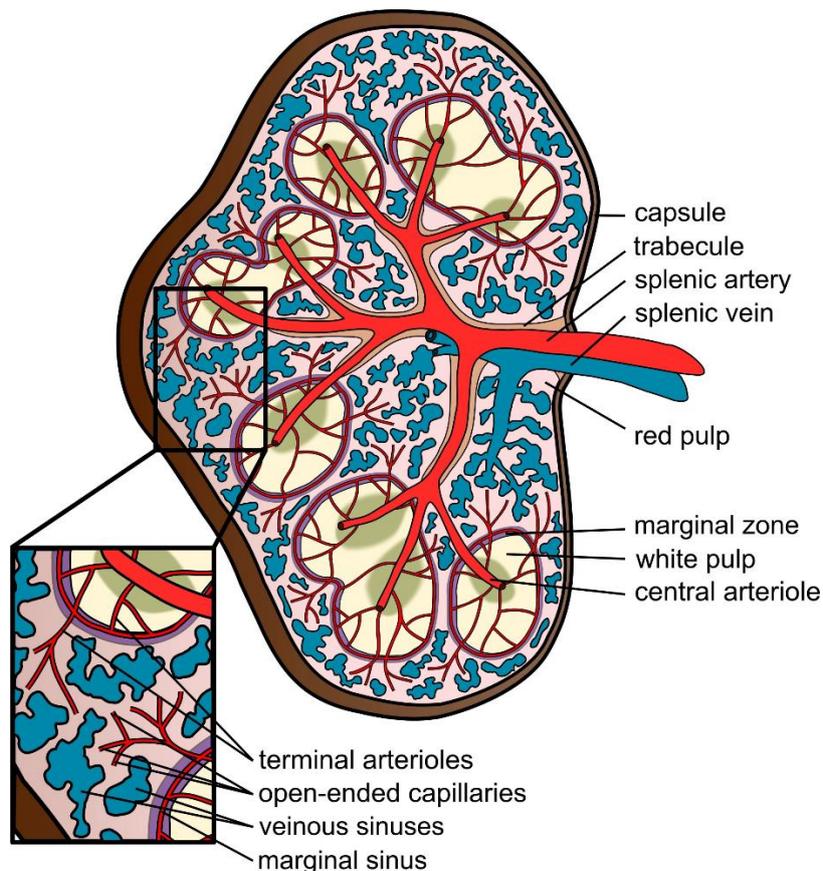


Figure 19. Structure of the spleen. The afferent splenic artery branches into central arterioles, which are sheathed by white pulp areas. Arterioles end in cords in the red pulp, from where the blood runs into venous sinuses which collect into the efferent splenic vein. From Bellomo et al, 2021¹¹⁵.

3. Principles of hemorheology

The word « hemorheology » comes from the ancient Greek as “hemo” which means “blood”, and “rheo” which means “to flow”. In general, rheology is defined as the study of the flow and deformation of matter, which describes the interrelation between force, deformation, and time. Thus, the meaning of hemorheology is the biophysical behavior of blood under flowing conditions¹¹⁶.

One of the key parameters of blood rheology is blood viscosity. Blood viscosity is defined as the ratio of shear stress on the shear rate. The shear rate corresponds to the strain rate of the fluid, while the shear stress is the frictional force that is exerted in a parallel or tangential direction on the fluid. When fluid is ongoing a laminar flow, so when fluid layers slide in parallel with no disruption between the layers, blood viscosity can be calculated from shear rate and shear stress¹¹⁷, as previously mentioned.

The study of hemorheology focuses on blood flow and properties of RBCs, their interaction and behavior into the circulation, and their role in the regulation of tissue perfusion.

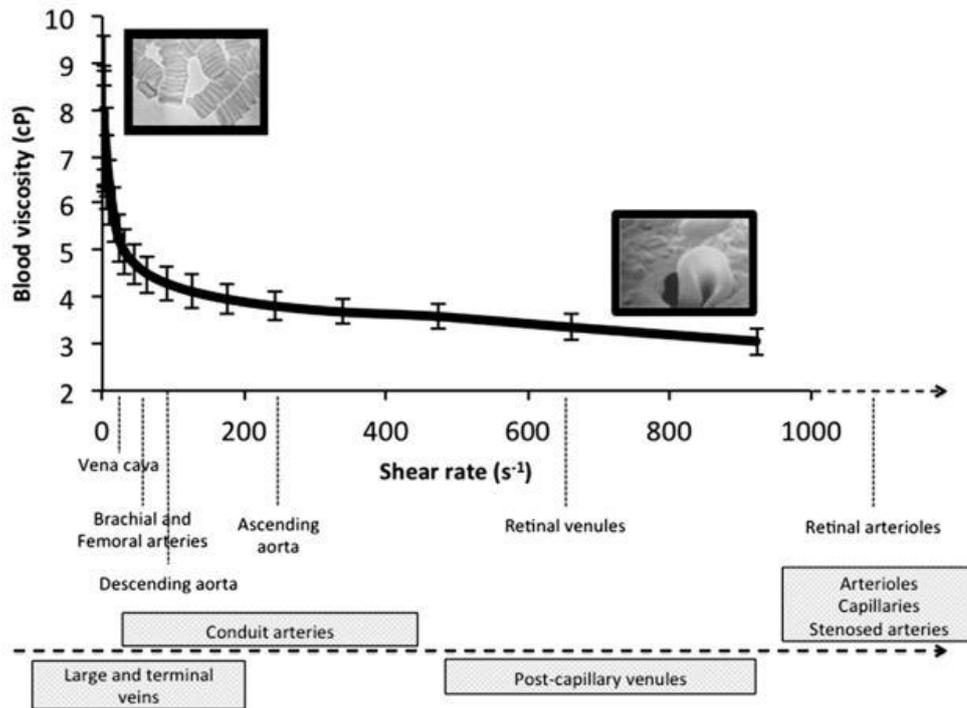
Blood is a two-phase liquid, composed of plasma and cellular components and its rheological properties depend on the flow properties of both phases. Several kinds of fluids can be found in the Nature: Newtonian and non-Newtonian ones¹¹⁸.

In Newtonian fluids, the viscosity remains constant independently of the shear rates and for a constant temperature. Examples of Newtonian fluids are water, mineral oil, and plasma. Indeed, plasma viscosity is independent of shear rate. In Non-Newtonian fluids, the viscosity depends on the shear rate/shear stress applied. Blood is a non-Newtonian fluid because its viscosity changes with shear rates¹¹⁹.

There are two types of non-Newtonian fluids:

- Shear-thinning fluids, like blood, in which the viscosity decreases when shear rate increases.
- Shear-thickening fluids, like the egg white, in which the viscosity increases when shear rate increases.

Because shear rate changes with the vessel diameter and blood flow velocity (shear rate = $4Q/\pi r^3$ with Q = volumetric flow rate and r = radius of the tube), blood viscosity changes over the vascular system. Indeed, blood viscosity is different in arteries, venules, and capillaries (Figure 20).



- **Figure 20.** Blood viscosity changes depending on shear rates. RBCs tend to form aggregates at low shear rates. Then RBCs aggregates are disaggregated when the shear rate increases, making blood viscosity lower than at low shear rates. From Connes et al, 2016¹²⁰.

3.1 Blood Flow and vascular resistance

The vascular system plays a significant role in the regulation of blood flow in the body. The cardiovascular system is made of “tubes” (arteries and veins) full of liquid (blood) connected by a “pump” (heart).

Flow velocity in a tube depends on pressure and flow resistance; from the Poiseuille equation¹¹⁹, we know that flow resistance (R) depends on the length (L) and radius of the tube (r) and fluid viscosity (η):

$$R = \frac{8\eta L}{\pi r^4}$$

Thus, the movement of a fluid is driven by a difference in pressure, flowing from a higher to lower pressure. Even if this equation helps to understand the flow of a liquid through a tube system, it can't be strictly applied to the cardiovascular system and blood flow¹²¹. In a physiological context, the parameters of the equation are not independent of each other. Vessels and arteries are not rigid tubes, and they can modulate their diameters in response to physiological stimuli. Different molecules control peripheral resistances, such as metabolites (CO₂, K⁺, H⁺), endothelin 1, histamine, hormones (angiotensin, vasopressin), and Nitric oxide (NO)¹²². NO is one of the most important molecules which promotes vasodilation, and its synthesis is stimulated by the increase of shear stress (i.e., the product of blood viscosity by shear rate) which promotes the activation of endothelial NO-synthase through shear stress-dependent mechanisms¹²³. This causes compensatory vasodilation and decreases arterial pressure¹²⁴. Indeed, blood viscosity may directly modulate the diameter of vessels, impacting on the whole vascular resistance.

3.2 Effect of Hematocrit on Blood Viscosity

Blood viscosity depends on the number and volume of RBCs (i.e., hematocrit). The higher the hematocrit, the greater the blood viscosity¹²⁵. The effect of hematocrit on

blood viscosity is greater at low than at high shear rate, where high hematocrit also promotes RBC aggregation¹²⁶.

3.3 Plasma viscosity

Plasma is a Newtonian fluid, which means that its viscosity is independent on shear rates. Plasma viscosity mainly depends on the fibrinogen concentration. Increased plasma viscosity is characterized by elevated concentrations of acute phase reactants and inflammatory cytokines¹²⁷. Since a cell-free layer is flowing close to the vascular wall, the viscosity of plasma plays a key role in the modulation of the endothelial nitric oxide synthase activity through shear stress-dependent mechanisms¹²⁸.

3.4 Red Blood Cell deformability and aggregation

The structure of RBCs influences their rheological properties, and their deformability depends on the surface/volume ratio, viscous-elastic membrane properties, and the intracellular viscosity of the cytoplasm, which is mainly dependent on mean corpuscular hemoglobin concentration (MCHC) and the type of hemoglobin¹¹⁹. The alteration of at least one of these parameters can cause a decrease in RBC deformability, inducing their early elimination from blood circulation.

The rheological properties of RBCs influence their shear-thinning characteristics. At high shear rates, RBCs undergo a shape change and are forced to align in parallel with laminar flow streamlines. This phenomenon participates in decreasing the viscosity of blood when increasing shear rates and depends on the ability of RBC to deform. In diseases where RBCs are poorly deformable, such as in sickle cell disease (SCD), the viscosity of blood is usually higher than in the general population, for a given hematocrit¹²⁰. At low shear rates, RBCs lose their parallel orientation and start to form the so-called “rouleaux” structure, reminding a bi or three-dimensional stack of coins¹²⁹. The aggregation or disaggregation process of RBCs is reversible¹³⁰, and it depends on several factors, such as the shear rate/shear stress applied to the cell suspension, cell geometry and deformability, cytoplasmatic composition and plasma viscosity, membrane properties and their extracellular negative domains (mainly sialic acids¹³¹), which prevent cellular aggregation.

Some plasma molecules can promote RBC aggregation, such as fibrinogen, a plasma protein that also plays a key role in the cascade of coagulation. The fibrinogen concentration directly impacts on the size of RBCs aggregates: the higher the fibrinogen concentration, the greater RBC aggregates formation is observed¹³².

There are two main theories to try to explain the rouleaux formation of RBCs: the “bridging model” and the “depletion model”¹³⁰. In the first one, it is assumed that fibrinogen molecules form a “bridge” between two adjacent RBCs, inducing the formation of the “rouleaux” (Figure 21). In the second model, the macromolecules concentration at the RBC surface is lower compared to the concentration in the

whole bulk concentration. This exclusion of macromolecules near the cell surface leads to an osmotic pressure which pushes the RBCs against each other to aggregate¹³⁰.

In some hematological diseases, RBCs have a high aggregation rate, forming sticky rouleaux which requires the application of high shear rates to disaggregate them¹³³.



Figure 21. Red blood cells aggregate at low shear rate. From <http://home.ku.edu.tr/~ozlemyalcin/aggregation1.html>.

4. Red blood cell Disorders: Sickle cell Disease

4.1 Introduction

The term Sickle cell disease (SCD) refers to a group of genetic conditions called hemoglobinopathies, which are characterized by the synthesis of abnormal hemoglobin molecules.

SCD is characterized by the synthesis of an abnormal hemoglobin, due to a single point mutation in the β -globin gene located on chromosome 11. In the case of sickle cell anemia (SCA), a mutation causes a substitution of a glutamic acid by a valine at the sixth position of the β -globin chain, leading to the synthesis of an abnormal hemoglobin, called Hemoglobin S (HbS)¹³⁴. HbS polymerizes under deoxygenation and forms filamentous tubular structures, inducing a mechanical distortion of RBCs and giving the typical sickle shape to RBCs¹³⁴. SCA is an autosomal recessive disease affecting red blood cells, so when individuals inherit one mutated copy of the β -globin gene, they are heterozygous for the β^S -allele and are called sickle cell trait (HbAS) carriers (Figure 22 A). Sickle cell trait is considered to be an asymptomatic condition although some authors reported that sickle cell trait could increase the risks of some cardiovascular complications, notably when associated with diabetes¹³⁵. When individuals inherit two β^S -alleles, they are homozygous recessive, and they have SCA.

Another specific defect in the β -globin gene causes another structural variant of hemoglobin, called hemoglobin C (HbC). HbC is the result of substitution of lysine for glutamic acid in the sixth position of the β -globin chain (Figure 22 B), which induces an electrostatic interaction between positively charged beta-6-lysyl groups and negatively charged adjacent molecules¹³⁶. This mechanism reduces HbC solubility in RBCs and causes the crystallization of HbC in both oxygenated and deoxygenated conditions and promotes RBCs dehydration¹³⁷, decreasing the lifespan of RBCs and increasing blood viscosity. People with hemoglobin C trait (HbAC) are phenotypically

normal. Those with two β^C -alleles are also, surprisingly most of times phenotypically normal, although RBCs are severely dehydrated, rigid and blood viscosity is very high¹³⁸. Individuals who inherit one HbC variant and one HbS variant, have sickle cell HbSC disease, which represents the second most common type of SCD¹³⁹. In that case, HbC promotes dehydration of RBCs, which in turn increases the relative amount of HbS inside RBCs, increasing the tendency of HbS to polymerize under deoxygenation.

In contrast to healthy RBCs, sickle RBCs are more rigid¹⁴⁰ and have increased adhesion properties¹⁴¹. For these reasons, they cannot easily flow in the vessels, especially in the microcirculation, and may trigger painful vaso-occlusive crisis (VOC). Moreover, sickle RBCs are more fragile than normal RBCs, which leads to high rate of intravascular hemolysis and chronic anemia¹⁴². Research works conducted in the last two decades have also demonstrated a key role of intravascular hemolysis in the development of chronic vasculopathy in SCD patients^{134,28}.

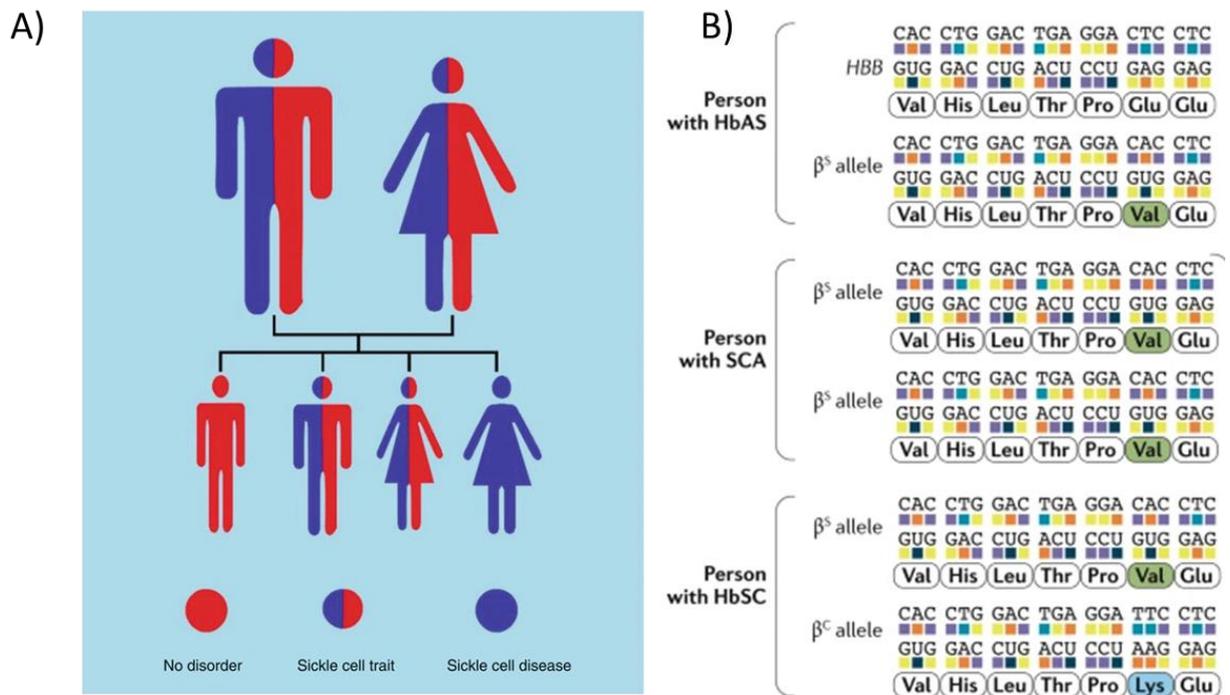


Figure 22. Inheritance of sickle cell trait and sickle cell disease (A), mutations and genotypes of sickle cell trait, SCA and sickle SC disease (B). Image edited from Al-Salem 2015¹⁴³ (A) and Kato et al, 2018¹⁴⁴ (B).

4.2 History

The term “sickle” was introduced in 1910 in the USA by Dr. James B. Herrick, who described “peculiar elongated and sickle-shaped red blood corpuscles in a case of severe anemia”¹⁴⁵. In the next 15 years after its first description, other similar cases were reported, increasing the interest in this new disease. The relationship between the sickling of the cell and the low oxygen concentrations was first reported in 1927 by Hahn and Gillespie¹⁴⁶ and in 1957 the genetic mutation causing the disease was reported for the first time by Vernon Ingram¹⁴⁷. In 1978 the β -globin gene was isolated for the first time¹⁴⁸ and two years after, a prenatal screening test for SCD was optimized by Yuet Wai Kan¹⁴⁹.

4.3 Global prevalence

SCD is the most common inherited disease worldwide; it affects millions of people all over the world with about 300.000 babies born every year¹⁵⁰. It is particularly common among individuals whose ancestry came from endemic areas for malaria, such as sub-Saharan Africa, the Caribbean, central Africa, India, and Mediterranean area¹⁵¹. Due to the migrations of these populations, the disease spread to other countries, such as in the Americas and Western Europe. More recent migrations episodes from French colonies (slavery period) have spread the disease in France too, and today sickle cell disease is the first genetic disease in France¹⁵² (Figure 23).

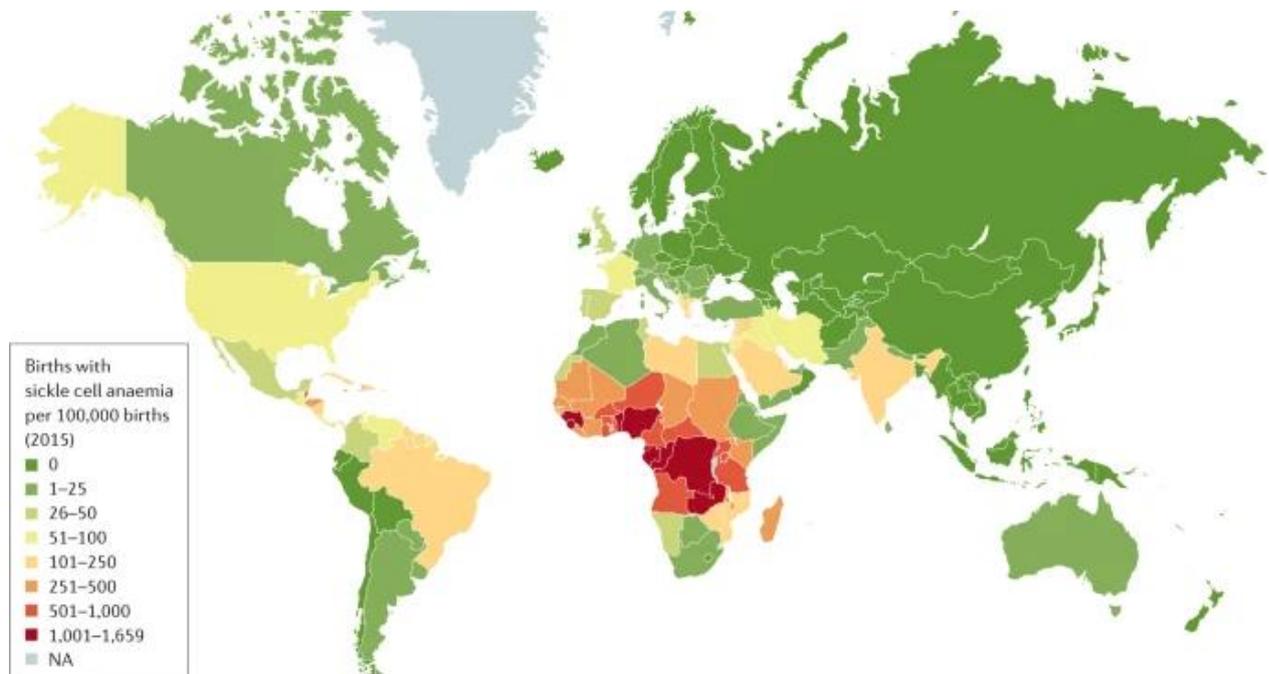


Figure 23. Incidence of sickle cell anemia. Estimation per 100.000 newborns with SCA in 2015. From Kato et al, 2018¹⁴⁴.

4.4 The malaria hypothesis

The epidemiology of SCD is strictly linked to the past and present distribution of Malaria, an infectious disease caused by the parasite *Plasmodium Falciparum*. The parasite is spread to humans through the bites of infected *Anopheles* mosquitoes, and it causes serious infections which can be fatal¹⁵³. People who get malaria have high fevers and hemolytic anemia, which is due to the lifecycle of the parasite inside the RBCs¹⁵³.

Despite the abnormal properties of HbS, this hemoglobin confers protection from severe malaria. While homozygous individuals (HbSS) for the mutation in the β -globin gene are at risk for developing vascular complication and VOC, sickle cell trait carriers are partially protected against severe malaria¹⁵⁴. For this reason, malaria is supposed to be an evolutionary arm of the host against the parasite. The first association between SCD and malaria resistance was observed by Haldane in 1949 and several studies have been published during the following years confirming this association¹⁵⁵.

Different hypotheses have been formulated to explain the HbAS malaria resistance, including the phagocytosis of sickled and infected cells, premature hemolysis causing parasite death, and inhibition of the parasite growth in RBCs exposed to low partial O₂ pressure¹⁵⁶. However, despite the epidemiologic evidence of a selection for sickle

cell trait carriers, the molecular mechanism protecting against severe malaria in this population is still unclear¹⁵⁷.

4.5 Sickle cell Disease pathophysiology

4.5.1 HbS polymerization

The polymerization of sickle hemoglobin is the primary event in the pathophysiology of SCD. The single point mutation which causes SCD, and more particularly SCA, is responsible for the substitution of negatively charged glutamic acid by the hydrophobic valine at the sixth position of the two β -chains of hemoglobin. In its T-form, so in deoxygenated conditions, HbS exposes this hydrophobic valine on the surface which interacts with the β^{88} leucine, β^{85} phenylalanine, and β^{73} aspartic acid residues on adjacent HbS molecules. These hydrophobic interactions promote nucleation and alignment of HbS molecules into rigid microfibrils, which deform the RBC conferring the typical sickle shape (Figure 24).

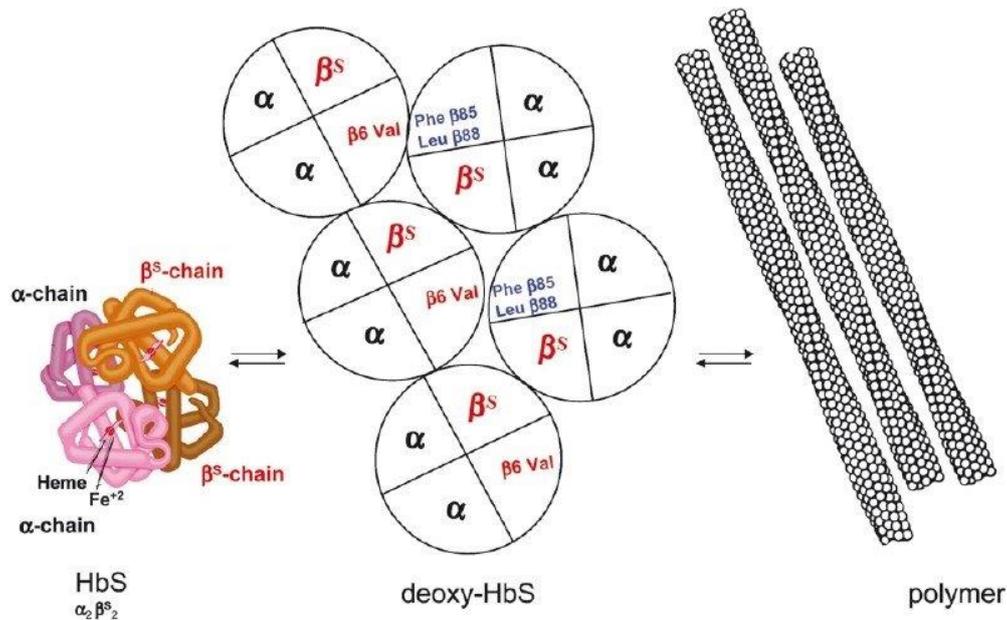


Figure 24. Structure and Polymerization of Hemoglobin S (HbS). HbS aggregates in deoxygenated conditions, forming polymers that give to the red cell the sickle shape. From Odièvre et al, 2011¹⁵⁸.

4.5.2 The kinetics of polymerization of HbS

HbS polymerization is influenced by several factors such as the HbS concentration into RBCs and the HbS affinity for oxygen¹⁵⁹. All the conditions that impact on these two factors can promote HbS polymerization. As discussed below, the affinity of HbS for oxygen is influenced by several parameters such as the 2,3 DPG concentration, temperature, and CO₂ level¹⁶⁰.

HbS polymerization takes place in two phases, which is called “double nucleation”. The main idea of this mechanism is that the first phase called “nucleation” is composed of the interaction of a few molecules of HbS, forming a little “nucleus” of HbS. The progressive addition of other HbS molecules to this nucleus is called “growth” and it reaches a critical point called “secondary nucleation” (Figure 25) in

which new fibers polymerize on the surface of existing ones forming a bigger and more stable HbS structure¹⁶¹.

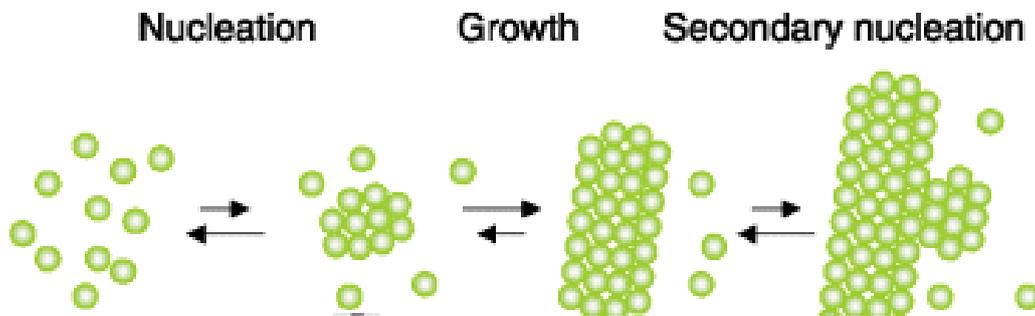


Figure 25. HbS polymerization model through the double nucleation process. Homogenous nucleation of HbS polymer and second heterogeneous nucleation. From Vekilov et al, 2007¹⁶².

The delay of polymerization depends on the speed of deoxygenation, which may impact on the shape of sickle RBCs. When HbS polymerization occurs slowly, the formation of fibers is homogenous, and they grow in one direction giving RBCs the typical sickle shape. When the polymerization is faster, the fibers formed are shorter and RBCs do not appear sickled, having a nearly normal disc shape even though they contain multiple domains of polymerized HbS hemoglobin¹⁶³ (Figure 26).

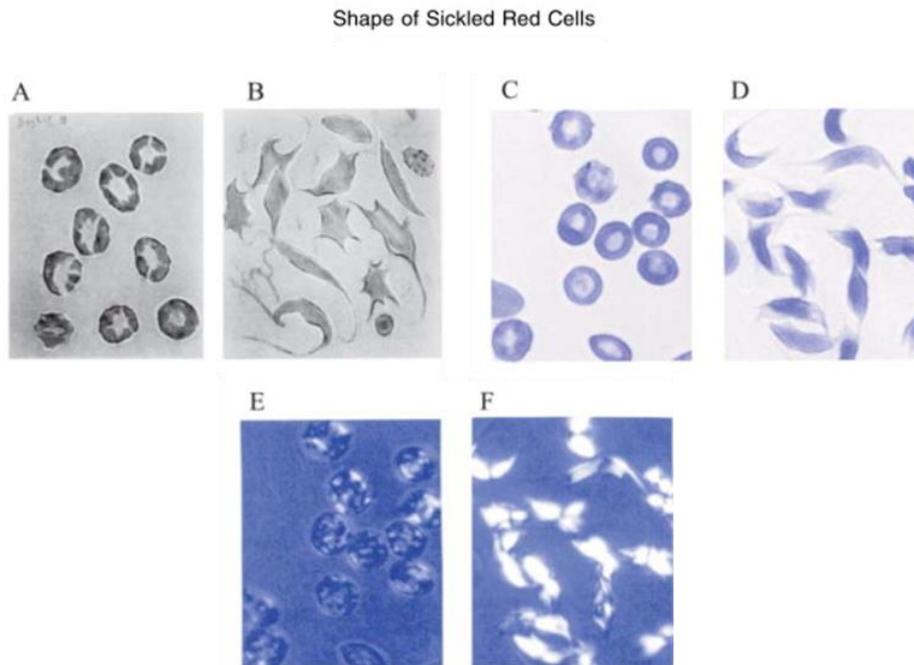


Figure 26. Sickle RBCs shape is influenced by the speed of deoxygenation. Optical micrographs showing different sickle RBCs shapes after rapid (image A, C, E) and slow deoxygenation (B, D, F). From Christoph et al., 2005¹⁶⁴.

HbS polymerization depends on the affinity of HbS for oxygen. The production of 2,3-DPG during the glycolysis process influences the affinity of HbS for oxygen because 2,3-DPG binds to the center “pocket” of hemoglobin stabilizing the T-state (deoxyhemoglobin) and allowing it to exist without quickly converting to the R-state (oxyhemoglobin)¹⁶⁵. Thus, by binding to HbS, 2,3-DPG decreases the hemoglobin affinity for oxygen, shifting the oxygen-binding curve to the right. The oxygen affinity of HbS is the same as that of hemoglobin A, but blood of sickle cell patients exhibits decreased affinity for oxygen due to the increased concentration of 2,3-DPG in sickle red blood cells, compared to normal erythrocytes¹⁶⁶. The elevated 2,3-DPG levels is also responsible for the lower intracellular pH of the sickle red cells¹⁶⁷, which in turn moderately decreases the oxygen affinity of SS blood¹⁶⁷. The *in vitro* variation of pH

(in a physiological range from 7.2 to 7.4) has been reported to be correlated with a decreased oxygen affinity of whole blood from sickle cell patients, suggesting that HbS is more affected by acidosis than HbA, which increases the risk of sickling¹⁶⁷. This mechanism could be caused by the Bohr effect, since we know that the pH modulates the oxygen saturation. In physiological condition, so in healthy individuals, this could be beneficial in some tissues, such as in skeletal muscle during exercise, promoting the release of oxygen. However, in pathological condition such as in SCD, acidosis would delay the time during which the HbS is in a deoxygenated state, promoting HbS polymerization¹⁶⁸.

The temperature can also influence the affinity of HbS for oxygen. Increased temperature decreases the affinity of HbS for oxygen¹⁶⁹. Indeed, the oxyhemoglobin is exposed to a higher temperature in tissues with a high metabolic activity where oxygen is required, thus the affinity decreases and hemoglobin unloads oxygen¹⁷⁰. The increased concentration of CO₂ is also correlated to an increased propensity of HbS to polymerize, mainly due to the effect of CO₂ on the pH of the blood¹⁷¹.

4.5.3 The effect of fetal hemoglobin

As previously introduced, HbF is the predominant type of hemoglobin present during gestation, and it is replaced by Hemoglobin A one or two months after birth. In SCD, HbF is replaced by the mutated HbS and for this reason, the clinical manifestations of

SCD are generally not evident until the switch from HbF to HbS, around the sixth month of life¹⁷².

HbF is an important modulator of the hematological profile and clinical expression of SCD. Indeed, HbF has a protective effect on the HbS polymerization because it can intercalate among the deoxy HbS polymers inhibiting the deoxy HbS polymerization and leading to the decrease of RBC rigidity¹⁷³. This beneficial effect of HbF is also reflected in the improvement of the rheological properties of sickle RBCs, a decrease of RBC oxidative stress and lipid peroxidation.

4.6 Deoxygenation and cation permeability of sickle Red Blood Cells

In the case of SCD, the mechanical distortion of RBCs caused by deoxygenation leads to an increased cation permeability, which can amplify the HbS polymerization and the cell dehydration in a vicious circle promoting sickling.

Increased RBC cation permeability has been shown to play a key role in SCD pathophysiology through its effects on RBC hydration status, HbS polymerization and sickling. The KCl cotransporter (KCC) mediates coupled efflux of K^+ and Cl^- leading to dehydration¹⁷⁴. The KCC activity is abnormally elevated in RBCs from HbSC patients, and it can be triggered by low pH, cell swelling, and urea. The KCC activity can remain activated during deoxygenation, leading to Mg^+ efflux and increasing its activity through cell dehydration.

The interest in the importance of the electrogenic Ca^{2+} uptake pathway (the so-called P_{Sickle}) has been acknowledged¹⁷⁴. However, its molecular identity remains debated.

Different cation channels mediating the Ca^{2+} influx could contribute to the P_{Sickle} , such as voltage-activated non-selective cation channels, NMDA¹⁷⁵ receptors, and Piezo1¹⁷⁶.

The P_{Sickle} is the Ca^{2+} influx into RBCs, which activates the Gardos channel, leading to a K^+ release from the cells and resulting in Cl^- and H_2O efflux.

This mechanism results in red cell dehydration and in increased HbS intracellular concentration, which in turn polymerizes and causes the mechanical distortion of the cell and triggers the cation permeability (Figure 27). As already explained, Ca^{2+} influx into RBCs may also promote senescence and the shedding of microvesicles from the RBCs membrane.

Indeed, increased P_{Sickle} activity and consequent Ca^{2+} influx could also be accompanied by RBC senescence. The gain of function and increased activity of the Piezo1 channel are involved in hereditary Xerocytosis, another RBC disease caused by an increased Ca^{2+} permeability, cellular fragility, and propensity to hemolyze¹⁷⁷.

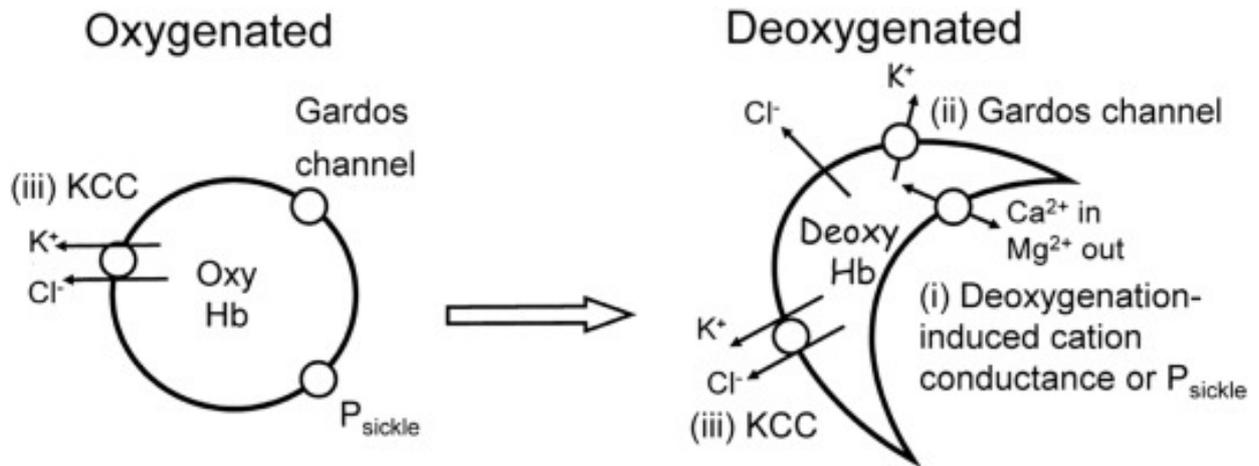


Figure 27. Cation permeability and dehydration of sickle RBCs in normoxia and hypoxia conditions. From Hannemann et al, 2015¹⁷⁴.

The massive Ca²⁺ influx into sickle RBCs and their higher intracellular levels compared to healthy RBCs have been associated with an increased NMDA receptors activation and Ca²⁺ conductance¹⁷⁸. It has also been reported that SCD patients with increased activity of Piezo1 channel due to the presence of the E756del allele (i.e., an allele responsible for a gain of function of Piezo1¹⁷⁹, had increased RBC dehydration and more dense RBCs compared to SCD patients without the E756del allele variant¹⁷⁹. These findings suggest that Piezo1 could modulate RBC sickling process through its effects on Ca²⁺ influx¹⁷⁹.

4.7 Blood rheology of sickle red blood cells

Sickle red blood cells show abnormal rheological properties, they are less deformable and more fragile compared to healthy RBCs¹²⁰. The cellular dehydration and the

subsequent increase of intracellular viscosity participates to this loss of RBC deformability. In addition, the repeated sickling/unsickling phenomenon leads to permanent membrane damages, which are responsible for the alteration of the rheological properties of sickle RBCs¹¹⁹. The chronic anemia in SCA patients is at the origin of the lower blood viscosity compared to healthy individuals. However, if the hematocrit is raised in experimental conditions to normal values, around 40%, SCA patients show higher blood viscosity compared to healthy donors because of the presence of irreversibly dense sickled RBCs and of RBCs with poor deformability (Figure 28)¹²⁰. This phenomenon is increased in deoxygenated conditions, when the decrease of partial O₂ pressure promotes RBC sickling¹⁸⁰. HbSC patients have higher blood viscosity compared to HbSS subjects at native hematocrit (but not at corrected hematocrit; Figure 28), but they have a lower risk of VOC maybe because of a better vascular function compared to HbSS patients¹⁸¹. However, increased blood viscosity in HbSC patients seems to be associated to an increased risk of otologic disorders and hypertension¹⁸² and proliferative retinopathy¹⁸³.

Plasma viscosity of SCA patients at steady state has been reported to be increased, probably due to the increased levels of circulating fibrinogen and other acute phase reactant proteins¹⁸⁴.

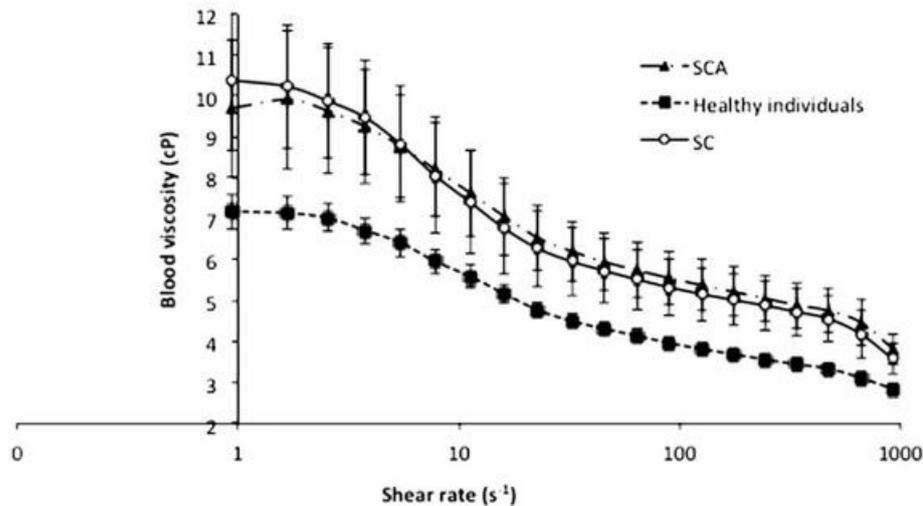


Figure 28. Blood viscosity of SC, SCA, and healthy individuals at the same hematocrit and different shear rates. From Connes et al, 2016¹²⁰.

It has been widely established that sickle RBCs are less deformable than normal RBCs; however, there is a variability in the deformability of sickle RBCs which can change during the life of the patient due to external factors such as blood transfusions, clinical conditions, or medications (Hydroxyurea)¹⁸⁵. RBC deformability is higher in young SCA patients (less than 5 years old) compared to older ones, due to the presence of HbF and it gradually decreases several months/few years after birth, when the HbF is replaced by HbS (Figure 29). However, the interest in sickle RBC deformability is not just about how it changes during the growth of the patient, but most of all how it changes during clinical complications and/or how it could predict the occurrence of clinical complications. The two models of pathophysiology of SCA, the hemolytic-endothelial dysfunction phenotype and the viscosity–vaso-occlusion one, are characterized by two different RBC rheological profiles. SCA patients with

higher RBC deformability at steady state and not under HU treatment are much more likely to develop frequent vaso-occlusive crises and osteonecrosis^{186,187}. This paradox is because more deformable sickle RBCs can easily adhere to wall vessels, promoting partial vaso-occlusions. This mechanism leads to the reduction of blood flow and prolongs the RBC transit time in a low-oxygen area, causing a decrease in the vascular lumen and promoting vascular occlusion¹⁸⁸. Moreover, patients with the highest RBC deformability have less fragile RBCs, resulting in higher hemoglobin level and blood viscosity compared to patients with more fragile RBCs¹⁸⁸. If blood viscosity reaches too high values, it may be a risk factor for frequent vaso-occlusive crises¹⁸⁶. Decreased RBC deformability, decreased hematocrit, and blood viscosity in SCA patients seem to be associated with the hemolytic phenotype, as the most rigid RBCs into the circulation are also the most fragile¹²⁰. The decreased deformability of RBCs has also been correlated to an increased aggregation force of RBCs, mostly reported in patients suffering from glomerulopathies¹⁸⁶, leg ulcers¹⁸⁹, and priapism¹⁹⁰. The findings of Bartolucci et al., also reported a higher number of dense RBCs in patients presenting leg ulcers, priapism, and renal dysfunction, in agreement with the hypothesis of lower RBC deformability in hemolytic phenotype¹⁹¹. The high rate of hemolysis which characterizes the hemolytic-endothelial dysfunction phenotype leads SCA patients to have lower hematocrit and blood viscosity and is responsible for the decreased bioavailability of NO. The main consequence of lower NO bioavailability is vascular dysfunction which in SCA culminates in clinical complications such as pulmonary hypertension, leg ulcers, priapism, and stroke. In

addition, free heme in the plasma is responsible for the production of ROS which in turn are NO scavengers¹³⁴.

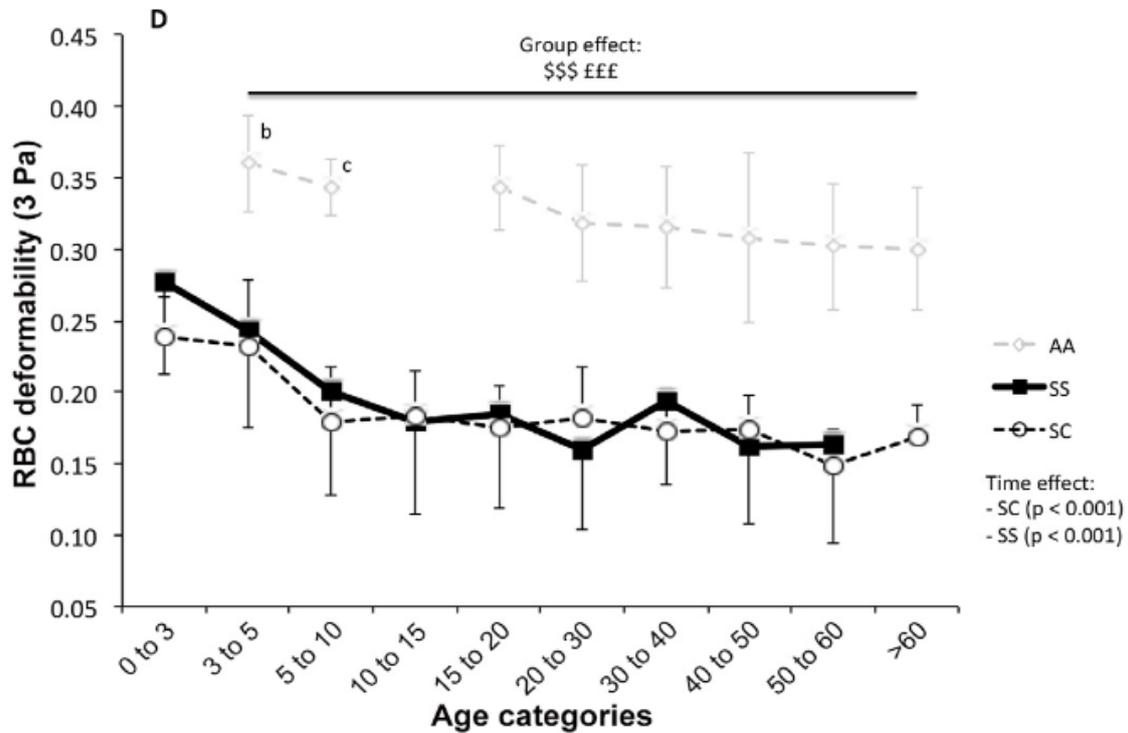


Figure 29. RBCs deformability at 3 Pa between AA, SS and SC patients at different age categories. From Renoux et al, 2016

One of the most challenging tasks in the study of SCD is the evaluation of the change of biological and blood rheological parameters during a vaso-occlusive episode. Despite this difficulty, several studies were done to evaluate the rheological properties of sickle RBCs just before and/or after a vaso-occlusive crisis. Ballas and Smith¹⁹² performed a longitudinal study of RBC deformability, percentage of dense RBCs, and hematological parameters in 36 SCA patients at steady-state and during painful crises. The results indicated that the painful crisis is a process whose rheologic characteristics follow a two-phase progression. During the first phase, the pain is

followed by a decrease in RBC deformability, and an increase in dense cell percentage. Interestingly, in some cases, the RBC changes may precede the clinical manifestation of pain by 1 to 3 days. A gradual improvement of the pain in the second phase of the crisis was followed by an increase in RBC deformability to values higher than those at steady state, probably due to the massive elimination of dense cells. The restoration of normal deformability profiles of sickle RBCs after a pain crisis was also correlated to a risk of developing a new painful crisis soon after the resolution of the previous one. This could be due to the fact that sickle RBC with higher deformability may expose a greater surface area to the endothelial cells, making them more prone to adhere to the vascular wall and impair blood flow into the microcirculation¹⁹².

Increased HbF levels (%), natural or induced by Hydroxyurea, improve the rheological properties and the clinical outcomes of both children and adults with SCA^{193,194}. The beneficial effect of HbF on the rheology of sickle RBCs is due to its effect on the inhibition of HbS polymerization, improving the deformability of sickle RBCs. This protective effect of HbF is also reflected by the decreased frequency of VOC in patients with higher levels of HbF¹⁹³.

The influence of alpha thalassemia on SCA has also been reported to improve the rheological and clinical profiles of patients¹⁹⁵. The effects of α -thalassemia on the rheological properties of sickle RBCs are more evident in patients homozygous for α -thalassemia ($-\alpha/-\alpha$), while they are milder for heterozygous patients ($-\alpha/\alpha\alpha$). Alpha-thalassemia causes a decrease in the mean cell volume (MCV) and MCHC, resulting in

a greater RBC surface/volume ratio and lower intracellular HbS, respectively. These changes result in a rise of RBC deformability¹⁹⁶. Moreover, Renoux et al., reported that the presence of alpha thalassemia modulates the levels of oxidative stress in young SCA patients, suggesting that the protective role of alpha thalassemia in SCA¹⁹⁷ could also be the results of lower oxidative stress-related RBC membrane damages. Despite these protective aspects, alpha thalassemia has been associated with higher risk of VOC in young SCA patients, which could be in part explained by the increased RBC deformability and aggregation of RBCs from alpha-thalassemia patients¹⁹⁸. The increased RBC deformability 1) may result in greater adhesion of RBC to endothelial cells and 2) results in lower RBC fragility and less hemolysis, which in turn lead to higher hematocrit and blood viscosity¹²⁰.

4.8 Red Blood Cell senescence in Sickle Cell Disease

As previously introduced, at the end of their lifespan, RBCs become senescent, and they are removed from blood circulation. Increased and early senescence of RBCs has been reported in several clinical conditions such as malaria, iron deficiency, diabetes, cancer.¹⁹⁹ Hemoglobinopathies, such as sickle cell disease and thalassemia²⁰⁰, have been associated with premature RBC senescence. Senescence of sickle RBCs is stimulated by increased cytosolic Ca²⁺ levels, which can reach 5 fold higher levels than those observed in healthy RBCs²⁰¹. The reason behind the higher Ca²⁺ levels is attributed to the P_{sickle} pathway, the increased cation permeability of sickle RBCs²⁰².

In previous works, the interest in the importance of the P_{Sickle} has been acknowledged, but its molecular identity remains unknown. The increased conductance of different cation channels mediating the Ca^{2+} influx has been reported in SCD, such as the NMDA receptors activity¹⁷⁸. Increased cytosolic Ca^{2+} may have several consequences, such as Gardos channel activation leading to K^+ , Cl^- , and water efflux from the cell and causing dehydration and shrinkage of the red cells²⁰³. Water loss in sickle RBCs also results in higher concentration of HbS, which in turn may promote RBC sickling. Two works also demonstrated that SCD patients with a gain of function mutation in Piezo1 had increased RBC density^{50,204}, suggesting that Piezo1 could play a role in RBC dehydration in SCD²⁰⁵. However, the direct proof of the contribution of Piezo1 in RBC dysfunction, RBC rheological impairment and sickling in SCD has not been provided until now.

High intracellular Ca^{2+} levels may cause a disruption of phospholipid asymmetry at the membrane level, through its effects on scramblase and floppase activities, that could lead to increased PS exposure on the RBCs surface²⁰⁶. SCD patients have higher percentages of circulating RBCs exposing PS than healthy individuals, which may bind to the endothelial cells of the vascular wall, compromising the microcirculation²⁸. Moreover, PS on sickle RBCs can also bind to platelets, promoting blood clotting and thrombotic events²⁰⁷. PS externalization is considered a hallmark of premature cell aging in SCD and the inefficient spleen activity to remove PS-positive RBCs contributes to the high number of PS-exposing RBCs in the circulation of SCD patients²⁷. Whether Piezo1 activation plays a role in advanced RBC senescence in SCD

is unknown, although the recent work of Chadebech²⁰⁸ suggests that it could be the case.

Increased cytosolic Ca^{2+} trigger membrane blebbing and microvesiculation, a mechanism by which the red cells try “to get rid of” the PS from the outer membrane to “prolong” the RBC’s life²⁰⁹. The production of microparticles (MP) has been reported to be increased in SCD, due to the higher intracellular Ca^{2+} levels associated with the disease¹⁰¹. RBCs-MP containing HbS-derived oxidation products, such as methemoglobin, heme, or free-iron can transfer these pro-oxidant molecules to the vasculature, increasing the oxidative stress-mediated pathophysiology of vascular dysfunction and apoptosis of endothelial cells²¹⁰. This mechanism may increase the expression of several adhesion molecules and modulate the inflammatory phenotype of endothelial cells through toll-like receptor 4 (TLR4) activation²⁸. Heme molecules are potent proinflammatory agents *in vivo* and they have been reported to trigger inflammation inducing Nod-like receptor nucleotide-binding domain leucine-rich repeat containing protein 3 (NLRP3) inflammasome activation in human endothelial cells²¹¹. NLRP3 inflammasome is an important component of the innate immune system that mediates the secretion of inflammatory cytokines IL-1 β /IL-18²¹² and its hyperactivation in SCD could contribute to chronic systemic inflammation.

High Ca^{2+} is not the only factor contributing to RBC senescence (Figure 30). The increased oxidative stress due to the high auto-oxidation rate of HbS may also promote accelerated RBC senescence. Lipid peroxidation and protein oxidation result in a loss of membrane lipid organization and decreased deformability of sickle

RBCs²¹³. The high levels of intracellular ROS may increase PS exposure on the outer membrane, promoting RBCs senescence, and adhesion and contributing to vaso-occlusions²¹⁴.

Several studies reported a correlation between increased senescence markers and clinical outcomes in SCD patients. Setty et al.²¹⁵ reported a correlation between the subpopulation of RBCs exposing PS and the risk of stroke in SCD, suggesting the role of PS in RBC-endothelium adhesion and its potential contribution to the phenomenon of vascular dysfunction and vaso-occlusion in SCD. Moreover, the correlation between the levels of circulating RBCs exposing PS and thrombin generation reported by Setty et al. indicates the prothrombotic properties of PS and its procoagulant activity²¹⁵.

The PS is expressed not only on the RBCs surface but also on microvesicles produced from the sickle RBCs membrane, suggesting that RBC-MP could participate in the alterations of vascular function in SCA. Besides, Garnier et al²¹⁶ demonstrated that microvesicles from SS patients treated with hydroxyurea were less capable to induce a pro-inflammatory phenotype in endothelial cells, compared to microvesicles collected before the Hydroxyurea (HU) therapy.

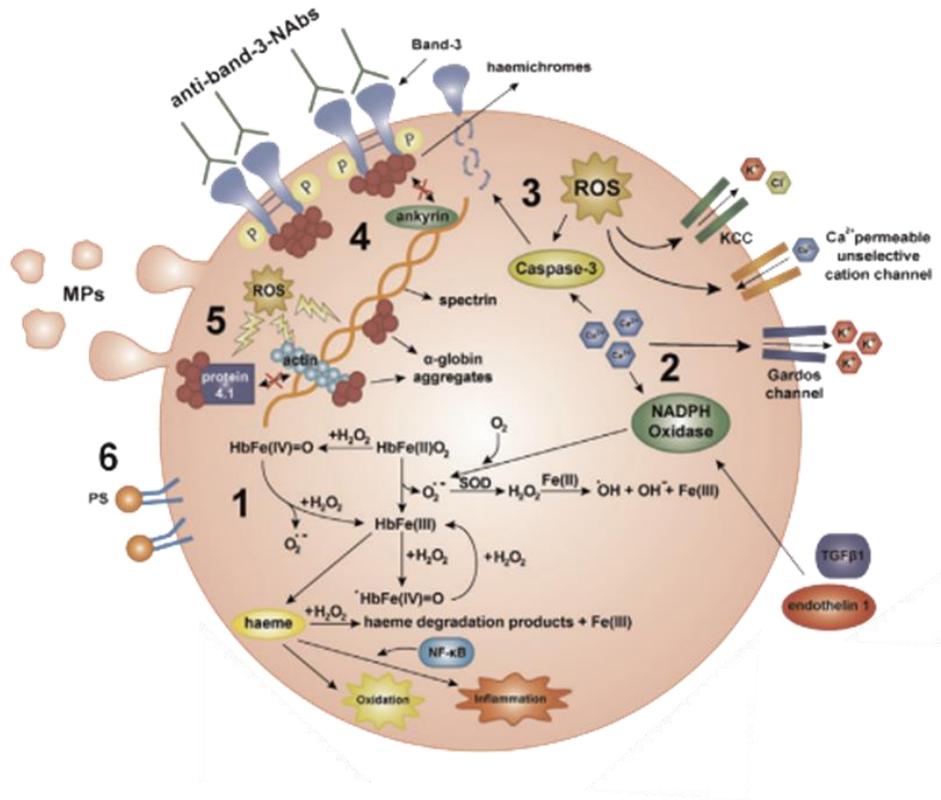


Figure 30. Different pathways participate to oxidative stress and premature senescence of RBCs in β -thalassemia and sickle cell disease. 1) Denaturation and auto-oxidation of Hb and formation of free heme and iron cause oxidative stress. The heme group is a pro-inflammatory molecule that activates NF-kb factor. 2) NADPH oxidase is overactivated by increased intracellular Ca^{2+} levels, and extracellular factors (proinflammatory cytokines). 3) The caspase 3 is activated by ROS and intracellular Ca^{2+} accumulation caused by the activation of non-selective cation channel stimulation. The caspase 3 degrades the Band3 and its junctions with the cytoskeleton. ROS may activate KCC channel causing K^+ and Cl^- efflux, and water loss. 4) Hemichromes bind to Band 3 causing its clusterization. 5) Intracellular ROS cause the oxidation of protein 4.1, destabilizing the cytoskeleton and the RBC membrane. 6) the alteration of the membrane is followed by the PS exposure on the outleaflet and membrane bubbling. From Voskou et al, 2015²¹⁷

4.9 Sickle red blood cells adherence and inflammation

The polymerization of deoxygenated sickle hemoglobin leads to cellular and membrane distortion and increases the propensity of the RBCs to adhere. This mechanism increases the formation of thrombus in the microcirculation which results

from the interaction between different cell types, such as dehydrated and dense sickle RBCs, young reticulocytes, leukocytes, platelets, and inflammatory cytokines²¹⁸. All these different types of cells can form cell-cell aggregates and/or bind to endothelial cells, causing their activation and leading to VOC. VOC are ischemic episodes in which tissues and organs in the affected area become deoxygenated, resulting in unpredictable and intense “pain crisis”²¹⁹.

The adhesion of sickle RBCs to the vascular wall and the sub-endothelial matrix is mediated by a large group of endothelial and cellular adhesion molecules. The activation of endothelial cells can be triggered by sickle RBCs exposing PS on their surface, which can be recognized by the PS-receptor on the endothelial cells, increasing adhesion and the risk of VOC. PS-dependent RBCs adhesion can be mediated via thrombospondin, a glycoprotein exposed by endothelial cells in response to endothelial injury (Figure 31). Manodori et al.²²⁰ demonstrated that pretreatment of RBCs with annexin V significantly decreased adherence by shielding PS on the RBCs. The PS exposure on the surface of sickle RBCs is also suggested to induce thrombin generation and activate platelets²¹⁵, reducing the effect of platelets’ cyclic adenosine monophosphate phosphodiesterase (cAMP), the inhibitor of platelets activation.

Sickle RBCs express other adhesion molecules in addition to PS, such as $\alpha 4\beta 1$ (at reticulocyte stage), Lutheran blood group and Basal Cell Adhesion Molecule (Lu/BCAM)²²¹, and intracellular adhesion molecule-4 (ICAM-4)²²², allowing the cells to adhere to and bind extracellular matrix proteins and counter receptors on the endothelium²²³.

Upon the lysis of RBCs, the liberation of cell-free hemoglobin and heme groups into circulation occurs.

Heme-exposed endothelial cells upregulate the expression of intracellular adhesion molecule-1 (ICAM-1), Vascular cell adhesion protein 1 (VCAM-1), P, and E selectin, due to increased exposition to oxidative stress²²⁴. Moreover, the role of the Toll Like Receptor 4 (TLR4) in heme-induced endothelial activation has been reported²²⁵, suggesting that its activation results in increased expression of ICAM-1, VCAM-1, P, and E selectin, but also in Interleukin-1 (IL-1), Interleukin-6 (IL-6), Interleukin-8 (IL-8) via Nuclear factor K-light chain enhancer of activated B cells (NF- κ B) production²²⁶. This mechanism suggests the role of released ROS in TLR4 signaling, which leads to oxidative stress and inflammation in a vicious circle.

The presence of free Hb in the plasma of SCD patients causes a lack of bioavailable NO, , leading to vascular rigidity and inflammation and expression of endothelial activation factors, including VCAM-1, ICAM-1 and endothelin-1²²⁷. The activation of endothelial cells is stimulated by proinflammatory cytokines produced by monocytes, such as Interleukin-1 β (IL-1 β) and Tumor necrosis factor α (TNF- α)²²⁸. This mechanism leads to a chronic inflammation state which in turn causes an increased expression of adhesion molecules²²⁹.

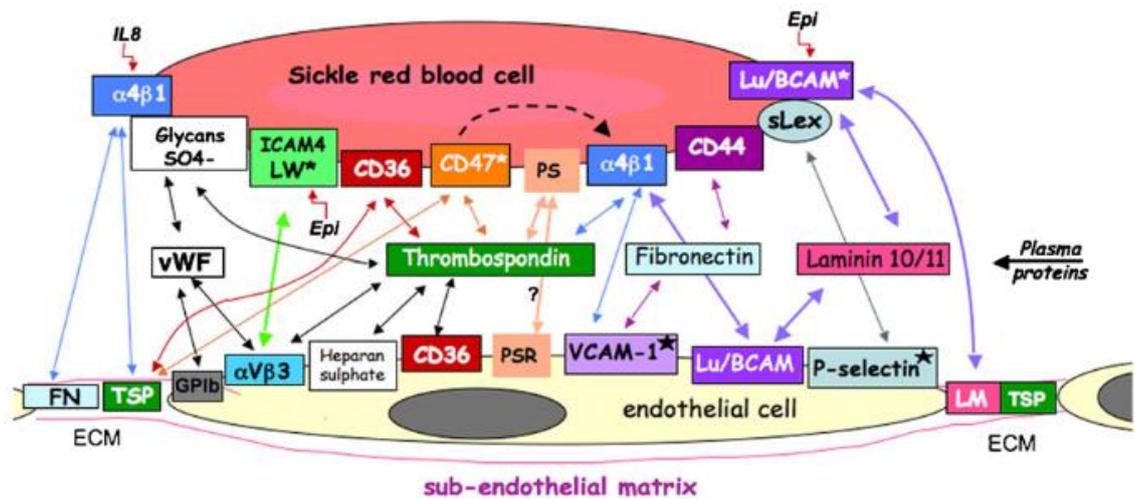


Figure 31. Major adhesive interaction pathways involved in the cellular adhesion mechanisms in SCD. From Cartron et al, 2008²³⁰.

A significant contribution to the “hypercoagulable state” and “inflammatory state” that characterize SCD comes from the MP, which are mainly produced from the membrane of RBCs²³¹ or activated platelets¹⁰¹.

MPs are small spheroid vesicles of 0.2 to 2.0 μm , containing mainly cytosolic and membrane-associated proteins (Figure 32). RBC’s MPs are present in low concentration in plasma from healthy individuals²³², but their concentration is increased in most cardiovascular diseases and in medical conditions characterized by hypercoagulability²³³.

In SCD, microparticles are shed from the sickle RBC membrane after repeated sickling/unsickling cycles and in presence of high intracellular Ca^{2+} levels. After their release, RBCs MPs may promote inflammation and oxidative stress, as they carry large amounts of hemoglobin, heme, and iron that may activate several signaling

pathways²¹⁰. In addition, RBC MPs have been shown to increase the adhesion of neutrophils to endothelial cells, through their effects on adhesion molecules expression²¹⁶. Nader et al, have shown that RBC MPs from SCD patients stimulated the expression of adhesion molecules on endothelial cells, as well as the production of pro-inflammatory cytokines through the activation of TLR-4²⁸.

Circulating MPs may also support coagulation activation by exposure of PS and tissue factor, promoting complex formation between coagulation factors in the coagulation activation cascade²³². The procoagulant state in SCD may also be due to the factor XI-dependent procoagulant properties of circulating RBC-derived MPs. Additionally, MPs have been reported to interact with factors Va, VIII, and IXa, facilitating assembly of prothrombinase complex^{234,235}.

MPs from sickle RBCs can induce the production of IL-6, TNF α , and IL-1 β in monocytes and they can transfer heme and iron to endothelial cells, causing vascular injury and potentially contributing to vascular dysfunction and vaso-occlusions.

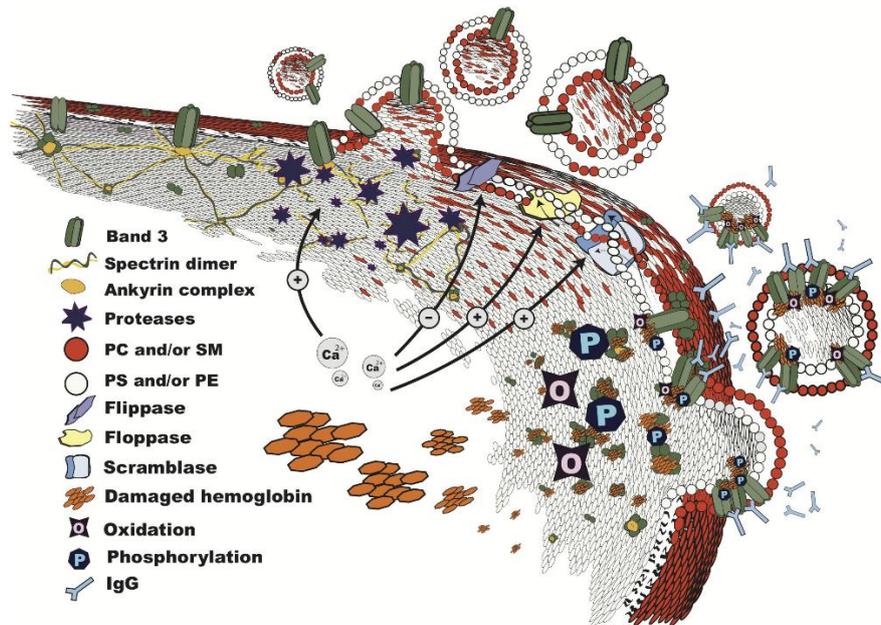


Figure 32. Red blood cell membrane during vesiculation. Disruption of the cytoskeleton, lipid bilayer rearrangement and production of microparticles exposing Band 3 and PS. From Leal et al, 2018²³⁶.

4.10 The role of Nitric oxide

Nitric oxide (NO) is an endogenous molecule with pleiotropic effects in different tissues. NO is synthesized by NO synthases, a family of enzymes that catalyze the production of NO from L-arginine and oxygen. There are three main NO-synthase isoforms: endothelial, neuronal, and inducible²³⁷.

NO is a major regulator of vascular tone and homeostasis, regulating several physiological properties of the blood vessels, such as vasodilation, vascular permeability, and antithrombotic properties²³⁸. NO is produced by endothelial vascular cells; it can rapidly diffuse to adjacent vascular smooth muscle cells (VSMCs) and activates the Guanylate cyclase. This mechanism causes the synthesis of 3,5-

cyclic guanosine monophosphate (cGMP), a molecule that induces the phosphorylation of proteins regulating the influx and efflux of Ca^{2+} in/from the sarcoplasmic reticulum and Ca^{2+} -dependent K^+ channels, promoting the relaxation of VSMCs (Figure 33). Low levels of NO produced by endothelial cells are sufficient to maintain the basal vascular tone²³⁸. In addition to vasodilation, endothelial NO has other physiological functions: it decreases the expression of adhesion receptors, inhibits platelet aggregation and modulates platelets-endothelial interactions²³⁹. In SCD, intravascular hemolysis causes the release of free hemoglobin, which interacts with NO produced from endothelial cells, causing the decrease of NO bioavailability and contributing to a loss of vascular reserve²⁴⁰. Free HbS in the plasma undergoes autoxidation, forming heme, iron and ROS which cause eNOS uncoupling and decreasing NO bioavailability¹³⁴. Other studies reported increased oxygen affinity²⁴¹ and a decrease of RBC sickling in response to NO^{242,243}. Intravascular hemolysis causes the release of arginase contained in RBCs into the plasma. Free arginase inhibits the production of NO through several mechanisms, including competition with NO synthase (NOS) for the substrate L-arginine, inducing the decrease of NO bioavailability²⁴⁴.

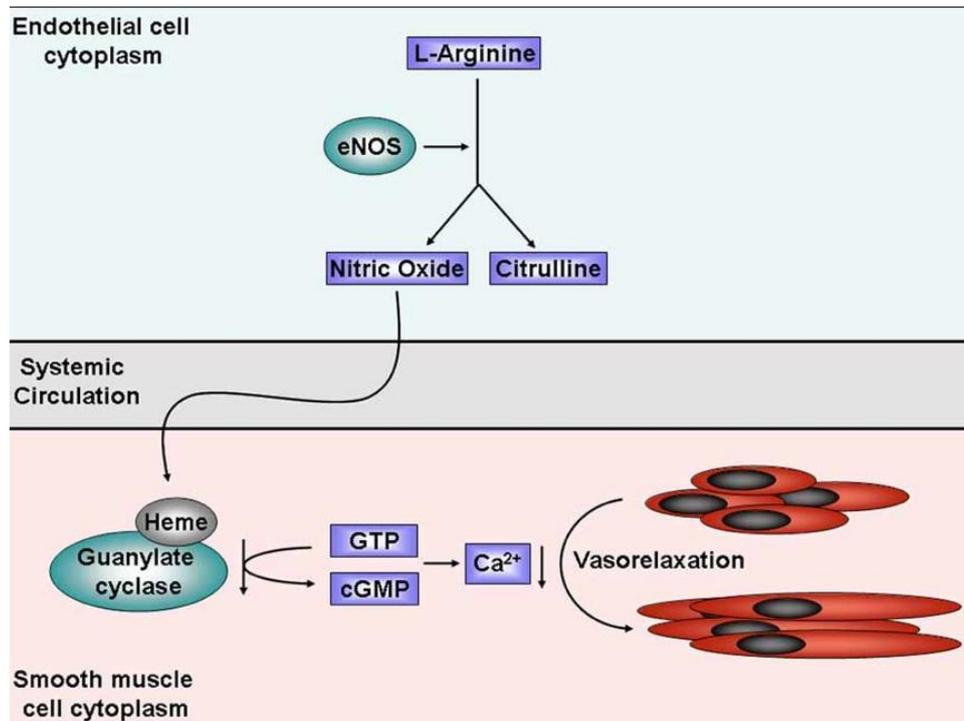


Figure 33. NO synthesis in endothelial cells and its effects on vascular smooth muscle cells. L-Arginine is converted to Citrulline and Nitric Oxide by the endothelial NO-synthase (eNOS). NO diffuses and activates the guanylate cyclase of vascular smooth cells, which increases the production of GMP-cyclase causing the Ca²⁺ efflux and relaxation of vascular smooth cells²²⁷.

4.11 Redox imbalance in Sickle Cell Disease

SCD is characterized by high levels of oxidative stress, which play a significant role in the pathophysiology of SCD-related microvascular dysfunction, vaso-occlusion, and the development of organ damages²⁴⁵. In general, oxidative stress is defined as the result of an imbalance between oxidants and antioxidants. Increased production of oxidants and/or decreased availability of antioxidants promotes oxidative reactions damaging lipids, proteins, and DNA (in nucleated cells), leading to premature cell death. In SCD, several mechanisms contribute to high oxidative stress levels, such as

the increased rate of auto-oxidation for HbS (compared to normal Hb), increased intravascular hemolysis, ischemia-reperfusion injury, and chronic inflammation²⁴⁵.

In presence of oxygen, HbS shows a greater tendency to auto-oxidation, inducing the formation of methemoglobin HbFe³⁺ from the oxidation of Fe²⁺ to Fe³⁺. This auto-oxidation leads to the production of free radicals within the RBCs, which are released in circulation in case of hemolysis²⁴⁶. The superoxide produced from HbS autoxidation dismutates into H₂O₂, a potential source of subsequent oxidative reactions. When H₂O₂ reacts with Fe²⁺, the hydroxyl free radical (OH·) is produced by the Fenton reaction. OH· triggers lipids peroxidation of the fatty acids on the red cell membrane, causing structural damage and promoting hemolysis²⁴⁷.

SCA patients with chronic hemolytic anemia show high levels of free HbS in the circulation. As previously introduced, free HbS reacts with NO reducing its bioavailability and leading to vascular dysfunction and acute inflammation²⁴⁸. The free Heme, given its hydrophobic nature, can insert into the membrane of endothelial cells promoting their activation, the production of ROS, and increasing inflammation²⁴⁵.

In SCA, high quantities of ROS are released in the circulation after repeated episodes of ischemia-reperfusion. Indeed, the hypoxia generated after vaso-occlusion and ischemia causes a hyper production of hypoxanthine and xanthine oxidase from adenosine triphosphate and xanthine dehydrogenase. When blood reperfusion

occurs, after the occlusion, the xanthine oxidase enzyme reaches the endothelium, increasing ROS production and reduction of NO²⁴⁹.

Among the several pathways leading the ROS production in SCD, there is the enzymatic generation of ROS by intracellular nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase and endothelial xanthine oxidase (XO). NADPH oxidase in sickle RBCs is regulated by Protein kinase C, Rac GTPase, and Ca²⁺ signaling, or by extracellular factors such as endothelin 1 and transforming growth factor β . NADPH oxidase contributes to the production of O₂^{•-} which causes direct oxidative damage to intracellular structures, reducing RBCs deformability and promoting RBCs fragility and hemolysis²⁵⁰. Xanthine oxidase is an enzyme present in high concentrations in endothelial cells. During inflammation, the oxidation of critical aminoacidic residues may occur leading to O₂^{•-} or H₂O₂ production at the endothelial surface²⁵¹.

4.12 Mitochondrial retention in sickle red blood cells

Recent studies on RBCs from SCD patients and murine models of SCD have demonstrated mitochondria retention in mature sickle RBCs. Jagadeeswaran et al.²⁵² reported that the mitochondrial retention in a SCD mice was associated with increased oxidative stress and that the pharmacological elimination of mitochondria leads to a decrease in oxidative stress. The abnormal presence of mitochondria in mature sickle RBCs would be the result of defective mitophagy genes⁸⁷. Martino et

al.⁸⁷ reported an association between mitochondria retention in mature sickle RBCs and decreased levels of mitophagy inducer proteins PTEN-induced kinase 1 (PINK1) and NIX, as well as elevated levels of Heat shock protein 90 (HSP90) chaperone in sickle RBCs. Moreover, the authors showed that the presence of mitochondria into RBCs was associated with increased PS externalization in SCD RBCs and hemolysis, suggesting a potential role of mitochondria in premature RBC senescence and fragilization⁸⁷.

However, defective mitophagy genes are not the only cause of the abnormal presence of mitochondria in red blood cells. Moras et al.⁸² first reported the essential role of the mitochondrial outer membrane protein voltage-gated anion channel 1 (VDAC1) in the terminal differentiation of human erythroblasts, which regulates the mitochondrial clearance of human terminal erythropoiesis. In this study, they showed that mitochondrial clearance begins with the transition from basophils to pleochroic erythroblasts and that downregulation of VDAC1 induces mitochondrial retention. However, one of the most challenging questions about the presence of mitochondria within sickle mature RBCs is about their functionality. Moriconi et al.⁸⁸ reported that red blood cells from one patients with SCD have functionally active mitochondria that can elicit a type 1 interferon response, suggesting that mitochondrial retention in RBC plays an important role in SCD complications, and it could lead to RBC alloimmunization. However, the functionality of residual mitochondria within sickle RBCs is still debated. Indeed, Martino et al⁸⁷. analyzed the characteristics of these sequestered mitochondria in sickle RBCs by

transmission electron microscopy (TEM) and they observed small mitochondria with a swollen and disorganized shape. Furthermore, they observed a lack of correlation between the presence of mitochondria and the amount of intracellular ROS. Together, these results led the authors to conclude that these residual mitochondria within sickle RBCs are not functional. These discrepancies in the literature leave the debate open.

A possible pathway triggered by the abnormal presence of mitochondria is illustrated in Figure 34. One could assume that the presence of functional mitochondria in sickle RBCs could increase intracellular oxidative stress, which could promote hemolysis leading to release of intracellular content in the circulation, including HbS, mitochondria, and free circulating mitochondrial-DNA (cf-mtDNA). Patient plasma may contain high levels of cf-mtDNA acts as potent pro-inflammatory molecules by triggering neutrophil extracellular traps (NETs). Moreover, cf-mtDNA have been reported to be hypomethylated and it could trigger the formation of NETs, acting as an erythrocytic Damage associated molecular patterns (DAMP)²⁵³. Free heme and free HbS released by hemolysis stimulate platelets thrombotic activation by inhibiting complex V of the platelet mitochondrial chain promoting an increase in mitochondrial ROS (mtROS) production (Figure 34).

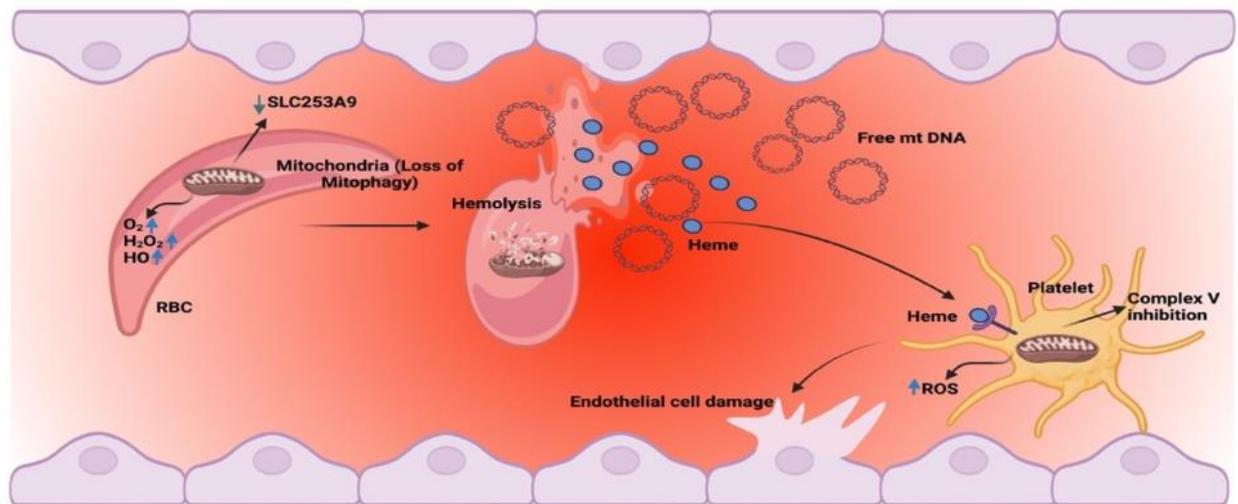


Figure 34. Hypothetic pathway triggered by the abnormal presence of mitochondria within sickle red blood cells. Release of free mt-DNA and heme in the circulation after hemolysis could cause platelets activation and endothelial cell damage. From Akhter et al., 2023²⁵⁴.

4.13 Sub-phenotypes and clinical manifestation

The phenotype and clinical complications are very different among SCD patients²⁵⁵. Disease outcomes depend on both genetic and non-genetic factors, such as the access to treatments and medications. Despite the high variability of clinical profiles among SCD patients, two distinct sub-phenotypes have been proposed: 1) Patients with a viscosity-vaso-occlusive sub phenotype and 2) patients classified in the hemolytic-endothelial dysfunction sub-phenotype¹³⁴ (Figure 35).

The viscosity-vaso-occlusive sub phenotype has been associated with higher hematocrit and hemoglobin concentration, and consequently increased blood viscosity^{256,120}, and reduced blood flow in microcirculation which may increase the

risk for painful vaso-occlusions, tissue ischemia, and reperfusion injuries. Bone marrow and periosteal infarction result in severe pain. The clinical manifestations of the viscosity-vaso-occlusive subphenotype are acute chest syndrome, osteonecrosis, and VOC.

The hemolytic-endothelial dysfunction sub-phenotype has been related to the rate of hemolysis, which would result in greater anemia and endothelial dysfunction²⁵⁷. Free Hb in the circulation reacts with NO, reducing its bioavailability and inducing vascular dysfunction²²⁷. Decreased Hb concentrations lead to increased reticulocyte counts, and higher total Bilirubin and serum Lactate dehydrogenase levels²⁵⁸. The main outcomes of the hemolytic-endothelial dysfunction subphenotype are pulmonary hypertension, priapism, leg ulceration, glomerulopathy and stroke/cerebral vasculopathy²⁵⁹.

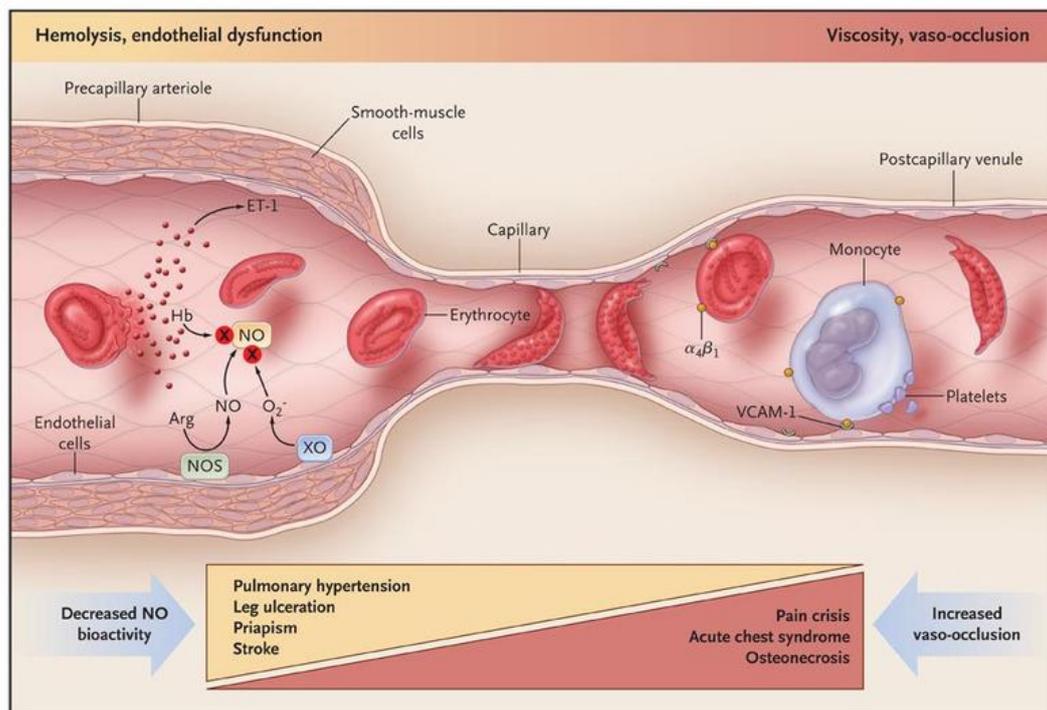


Figure 35. Pathophysiology, biological and clinical sub-phenotypes in sickle cell disease; from Gladwin et al., 2008²⁶⁰.

Of course, there is a co-existence of these two phenotypes and one patient can't be definitively classified into one clinical sub phenotype but studies show a trend for patients to develop more frequently some complications, depending on their biological phenotype ²⁵⁶.

4.13.1 Pulmonary hypertension

Pulmonary hypertension is a clinical complication due to the increase of blood pressure in the vessels that carry blood from the heart to the lungs (pulmonary arteries). A blood pressure ≥ 25 mm Hg at rest is defined as a pulmonary hypertension condition²⁶¹. This clinical complication is characterized by the remodeling of the pulmonary vascular structure and narrowing of the vascular lumen. In SCD, this complication is due to intravascular hemolysis which causes endothelial dysfunction and progressive pulmonary vasculopathy²⁶². Management of pulmonary hypertension in adult SCD patients is based on anticoagulation and hydroxyurea or transfusions²⁶³.

4.13.2 Renal failure

The renal complications of SCD also reported as glomerulopathy, are caused by RBCs sickling in the renal medulla and consequent vascular obstruction. The main consequences are hematuria, proteinuria, tubular disorder, and chronic kidney disease²⁶⁴. The medullary tissue promotes the sickling of RBCs because the oxygen partial pressure in this environment is very low (35-40 mmHg) and the osmolality, which promotes water reabsorption and urine concentration, also promotes the RBCs dehydration, increasing HbS concentration and propension to sickle.

The most common manifestation of renal damage is albuminuria, and it can be present in young or adolescent patients, indicating renal complications²⁶⁵. The evolution of the disease to chronic kidney complication can be very fast, and for this reason, hemodialysis treatments, or in extreme cases kidney transplantation, are suggested to treat the disease.

4.13.3 Priapism

Priapism is a prolonged and painful erection of the penis in the absence of sexual activity or stimulation²⁶⁶. It is a common complication in males with SCD including children and adults and it is caused by the obstruction of small vessels in the penis by sickle RBCs which stick together and block circulation¹⁹⁰. Management of priapism depends on its duration and the consequent penile ischemia, but it is essential to avoid the risk of impotence²⁶⁷. If it lasts less than 4h it can be treated with analgesics or painkillers²⁶⁶. If the duration is prolonged, a penile aspiration and drainage are

recommended, with the infusion of adrenergic agonists which mediates the smooth muscle relaxation. If the adrenergic treatment is not sufficient, a blood transfusion is carried out²⁶⁷.

4.13.4 Leg ulcers

Leg ulcers are a severe and chronic complication in SCD caused by poor blood circulation in the legs. They are painful and often disabling and usually happen on the lower part of the leg, in areas with less subcutaneous tissue and thin skin (tibia and malleolar region²⁶⁸). They usually progress in stages, getting larger and deeper into the skin²⁶⁸. Leg ulcers are more common in males²⁶⁹ and among patients with low levels of Hb and HbF²⁶⁹; their incidence increases with age²⁶⁹. Hemolysis, through its effects on NO bioavailability, would play an important role in the development of leg ulcers in SCD²⁷⁰. Treatments of leg ulcers include topical applications of analgesic and platelet-derived growth factor and may require administration of opioids in case of painful ulcers²⁶⁸.

Among the new medication for the treatment of leg ulcers in SCD patients, there is the topical sodium nitrite 2% ointment, a formulation which allows nitric oxide to dilate vessels by relaxing smooth cells. This mechanism helps healing the wound by improving the amount of oxygenated blood that reaches the leg ulcer and leading to a significant reduction in ulcer size²⁷¹. A Phase 1 clinical trial of this sodium-nitrite

cream demonstrated that the therapy is safe, well-tolerated, and significantly reduces ulcers pain in the patients enrolled²⁷¹.

4.13.5 Cerebral Infarction

Cerebral infarction is a major complication of SCD caused by the blockage of blood flow in the brain due to red blood cell sickling, resulting in tissue ischemia.

It is assumed that the vascular lesions are caused by the abnormal adhesion of sickle RBCs and leukocytes to the vascular wall, causing inflammation, hyperplasia of large vessels and silent progressive occlusion of the cerebral arteries²⁷²

It has been observed that the higher risk of the first ischemic stroke in HbSS patients is during the first ten years of age, especially between the 2 and 5 years old²⁷³. The incidence decreases in SS patients from 20 to 29 years of age and it increases again in older patients²⁷³.

Symptoms of stroke are headache, seizure, weakness, slurred speech, or confusion. Among the risk factors associated with ischemic strokes, there are prior transient ischemic attack (TIA), low steady-state hemoglobin concentration, rate of and recent episode of acute chest syndrome (ACS), and elevated systolic blood pressure²⁷⁴. While alpha thalassemia seems to lower the risk of having stroke in SCD, the role of G6PD deficiency is discussed with some studies showing that G6PD deficiency increases the risk of stroke while others did not find any association^{275,272,276}.

4.13.6 Vaso-occlusive crises

Vaso-occlusive crises (VOC) are the hallmark of SCD complications caused by obstruction of small vessels in microcirculation or intimal hyperplasia of large vessels, leading to hypoxia, ischemic injury, tissue damage and inflammation²⁷⁷. VOC are very painful complications, characterized by acute recurrent crises, chronic pain syndrome and neuropathic pain¹⁹¹. Nearly all patients with SCD experience VOC at some point, but the frequency is variable and unpredictable²⁷⁹. VOC occur because of multicellular adhesion of sickled RBCs to the vascular endothelium and their interaction with adherent leukocytes and platelets²⁷⁷. The propensity of sickle RBCs to adhere to the vascular endothelium has been reported to be associated to an increased RBCs deformability. Indeed, patients with high RBC deformability experienced more frequent VOC than those with low RBC deformability, suggesting that red cell deformability could be a rheological predictor of VOC¹⁸⁸.

4.13.7 Acute chest syndrome

ACS is a severe complication associated with disability and mortality among SCD patients. It is characterized by acute lung injury, respiratory manifestation, and lung infiltration, visible on lung imaging²⁸⁰. The pathophysiology of ACS is complex and involves vaso-occlusions in the pulmonary microvasculature, infection, and hypoventilation, accompanied by fever, chest pain, and tachypnea²⁸¹.

The main actors involved in the pathogenesis of ACS, are bone marrow ischemia and necrosis, which can lead to fat embolism²⁸¹..

The incidence of ACS is higher in children (2-4 years of age) than in adults (>20 years of age) with SCD²⁸². The outcome of the disease is usually milder in children, while adults' cases of ACS are more severe and have higher rates of mortality due to extensive bone marrow necrosis, which causes pulmonary fat embolism. Despite the differences between the groups, clinical management is similar in both children and adults²⁸¹. Management of ACS includes pain controls, antibiotics, supplemental oxygen, and blood transfusion. Pain control for both children and adult patients starts with Ketorolac, a nonsteroidal analgesic medication which provides pain relief without narcotic's risk of respiratory depression or hypotension²⁸³

Several hematological parameters are associated with the development of ACS. Rates of ACS are positively correlated with steady-state white blood cell count and Hb concentration and inversely correlated with HbF levels²⁸⁴.

4.13.8 Osteonecrosis

Osteonecrosis, also known as avascular necrosis or bone infarction, is a debilitating complication of SCD which affect about 30% of people with SCD²⁸⁵. It is caused by repeated ischemia-reperfusion of bone tissue due to lack of blood supply, leading to the death of hematopoietic stem cells of the bone marrow²⁸⁶. Hematopoietic cells are

very sensitive to low oxygen pressures and in case of a lack of blood supply, they die within 12 hours²⁸⁷.

Osteonecrosis mostly affects the femoral head, but many SCD patients also present a multifocal joint involvement²⁸⁸. The incidence of osteonecrosis is higher in adults with HbSS and co-existent alpha thalassemia²⁸⁹ showing high Hb concentration. In addition low white blood cell count, frequent VOs, and a history of ACS increase the risk for osteonecrosis²⁸⁵.

4.14 Diagnosis of Sickle cell disease

Early detection of SCD can help to reduce mortality and manage the disease²⁹⁰. Therefore, several techniques have been developed to detect the SCD and the carrier states, with high sensitivity and specificity²⁹⁰. Among the screening techniques, there are complete blood count, peripheral blood smears, solubility sickling tests, and hemoglobin electrophoresis^{290,291}. The confirmation of the disease can be done with genetic tests and if the diagnosis is confirmed, other tests to check for possible complications of the disease can be suggested.

SCD can be diagnosed in an unborn baby by sampling some of the amniotic fluid surrounding the baby in the mother's womb²⁹².

4.15 Treatment and medications

Management of SCD and the recognition of the main symptoms is essential to avoid pain episodes, relieve symptoms, and prevent complications. The medications for SCD are²⁹³:

4.15.1 Hydroxyurea

Hydroxyurea is the main medication used in SCD, which increases the production of Fetal hemoglobin. It was first tested in SCD in 1984²⁹⁴. Then, the United States Food and Drug Administration (US FDA) and the European Medicines Agency (EMA) approved hydroxycarbamide for the treatment of SCD in 1998 and 2007, respectively. As previously explained, HbF decreases the polymerization of HbS, and it has a protective effect on the pathophysiology of the disease. Based on this principle, hydroxyurea decreases the frequency of VOC, ACS, and transfusion needs. In a controlled trial testing the efficacy of hydroxyurea in SCA patients, Charache et al.,¹⁹³ observed a significant reduction in medial annual rate of painful crises, as well as a reduction in the frequency of ACS and number of transfusion. Hydroxyurea has been reported to ameliorate the rheological properties of sickle red blood cells, increasing the red cell deformability and decreasing the strength of RBCs aggregates²⁹⁵. For all this characteristics, Hydroxyurea treatment is consider to be the standard of care for all SCA patients, with a well-documented efficacy and a long-term safety profile¹⁹⁴.

4.15.2 Voxelotor

This molecule binds reversibly to hemoglobin, stabilizing the oxygenated hemoglobin state and preventing HbS polymerization by increasing hemoglobin affinity for oxygen. Patients with sickle cell disease ages 12 and older were shown to benefit from Voxelotor in Phase I/II and Phase III trials in terms of increasing hemoglobin concentration and decreasing hemolysis²⁹⁶. However, the molecule does not seem to provide a significant effect on VOC or a reduction in clinical consequences²⁹⁷, but decreases the severity of hemolysis and anemia in patients with SCD.

4.15.3 Crizanlizumab

This treatment consists of a humanized monoclonal antibody against P-selectin, which blocks the interaction between endothelium cells and platelets with erythrocytes and leukocytes. Through this mechanism, Crizanlizumab would prevent vaso-occlusion²⁹⁸. In November 2019 the FDA approved Crizanlizumab for reduction in frequency of vaso-occlusive crises. In October 2020, the EMA granted conditional Marketing Authorization for the use of Crizanlizumab in SCD patients aged 16 years and above (from <https://www.novartis.com/news/novartis-provides-update-phase-iii-stand-trial-assessing-crizanlizumab>).

However, preliminary results from the Phase III study showed no differences between the Crizanlizumab treatment and placebo, on the annual rates of vaso-occlusive crisis, leading to the withdrawal from marketing authorization in

Europe. (From <https://ansm.sante.fr/informations-de-securite/adakveo-crizanlizumab-retrait-de-lautorisation-de-mise-sur-le-marche-en-raison-dun-manque-defficacite>).

4.15.4 L-Glutamine oral powder

Recently approved by FDA, this drug would help in increasing the availability of reduced glutathione, suggesting its therapeutic potential as an antioxidant. A Phase-3 trial for L-Glutamine in SCD patients showed a reduction of median number of pain crises²⁹⁹. However, additional studies are required to establish an optimal or individualized dosing regimen and identify biomarkers of response to glutamine therapy³⁰⁰.

4.15.5 Preventing infections

Bacterial infections are a frequent cause of death in children with SCD. Penicillin administrations in children help to prevent infections such as pneumonia, which can be life-threatening to young patients. Childhood vaccinations are important for preventing disease in all children. They are even more important for children with SCA because their infections can be severe in case of splenectomy^{301,302}.

4.15.6 Painkillers

Pain crises are the most frequent clinical manifestations of the disease. Their frequency and intensity are extremely variable and require the administration of painkillers, such as paracetamol and opioids, whose administration requires sometimes the hospitalization³⁰³.

4.15.7 Transfusions

There are two different kinds of red blood cells transfusions: simple transfusions and automated exchange transfusions. A simple transfusion consists of the delivery of additional healthy red blood cells to the patient's body, while an exchange transfusion consists of the exchange of sickle-shaped red blood cells with healthy ones, reducing the number of sickle cells without increasing blood viscosity³⁰⁴.

Some risks and complications due to blood transfusion are present, including immune response to the donor blood, infections, and iron overload³⁰⁵. However, automated RBCs exchange is associated with significantly lower ferritin levels, compared to simple and manual exchange transfusions³⁰⁶.

4.15.8 Stem cell transplant

Today, allogeneic hematopoietic stem cells transplantation (HSCT) is the only cure for SCD and more than 1000 SCD patients have now received transplants worldwide until

today³⁰⁷. However, the hematopoietic stem cell transplantation carries a high number of risks of morbidity and early death. The bone marrow is the tissue where RBCs are produced and in a bone marrow transplant, the patient's bone marrow is previously treated and "destroyed" by chemotherapy, following the so called myeloablative conditioning regimen. Then, it is replaced by the bone marrow from a compatible healthy donor (generally brothers or sisters³⁰⁸). The use of Anti-thymocyte globulin (ATG), an antibody against human T-cells, was reported to decrease the rejection rate after the hematopoietic stem cell transplantation in patients with SCD ranging from 2 to 22 years of age³⁰⁹, indicating that the HSCT after myeloablative conditioning with ATG should be considered as a potential treatment for young SCD patients. This treatment is reserved for the most severe patients who do not respond to the other treatments, and the very high cost of the treatment represents another important limitation^{310,311}.

4.15.9 Gene therapy

Sickle cell disease is a genetic disorder and as such, gene therapy is the most advanced strategy to cure the disease. Even though it is very promising, gene therapy for sickle cell disease is currently not FDA-approved.

There are two main mechanisms gene therapy can tackle SCD, gene editing and the switching of silenced genes.

- 1) The gene editing is based on the use of the CRISPR-CAS9 enzyme, which can recognize the point mutation on the DNA and can cut and replace the mutation with the correct gene version³¹². This restores the ability of hemoglobin to bind oxygen and give RBCs back their normal shape (Figure 36).
- 2) The switching on silenced genes uses the CRISPR-CAS9 to remove the fetal hemoglobin repressor, BCL11A, from its binding site allowing the increase of HbF expression in erythroid progenitor cells³¹³. Clinical trials investigating the editing of BCL11A to reactivate HbF expression for SCD patients have preliminarily demonstrated disease symptom alleviation³¹⁴.
- 3) However, for lifelong benefits, the persistence of HbF expression is required, and more data are needed to determine the safety of the strategy³¹⁵.

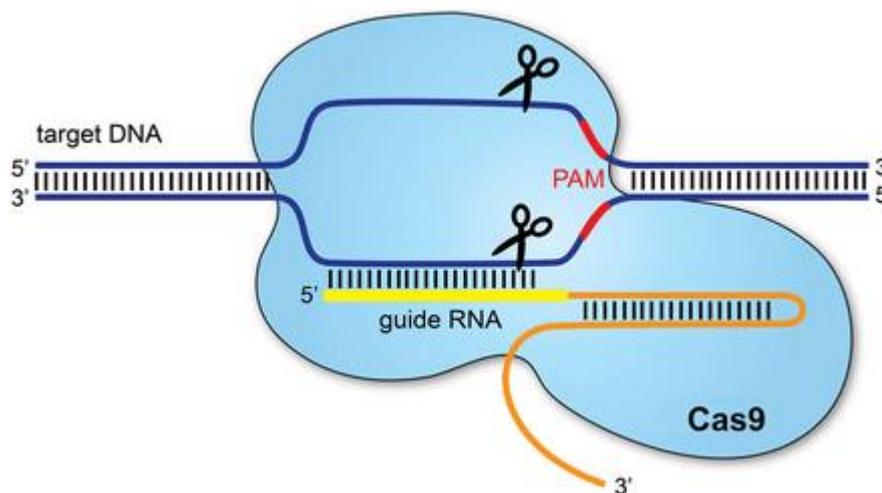


Figure 36 CRISPR/Cas9 gene-editing technology. The guide RNA matches a desired target gene and the endonuclease Cas9 cut the double-strand DNA to modify the genome. Figure from Redman et al., 2016³¹².

5. Aim of the thesis

The rheological and senescence properties of RBCs in SCD are influenced by the polymerization of HbS, as well as the repeated sickling and unsickling cycles. **However, high intracellular oxidative stress has also been shown to play a major role in the modulation of RBC properties in SCD^{28,316}.** RBC G6PD is essential to maintain normal levels of GSH, the main antioxidant in RBCs. Some patients have a coinheritance of G6PD deficiency and SCD, and this coinheritance has been shown to increase the risks for some complications, such as stroke²⁷⁵. **Whether the presence of G6PD-deficiency further deteriorate the RBC physiological/rheological properties of SCD patients is unknown.** Moreover, it has been suspected that mitochondria retention into mature RBCs in SCD could increase the generation of intracellular ROS and decrease RBC survival rate, but their functionality within sickle RBCs is still debated in the literature. If so, **the presence of mitochondria into RBCs should correlate with markers of RBC damages and impaired RBC rheology.**

The rheology of sickle RBCs and their propensity to sickle also depend on the hydration status of RBCs. The accumulation of Ca^{2+} into RBCs, notably during deoxygenation, promotes RBC dehydration through the activation of the Gardos channel (Psickle pathway)⁴². **However, no protein, or group of proteins, has been identified as being involved in the P-sickle pathway. We investigated whether Piezo1 and TRPV²⁺ modulation could impact on RBC sickling and senescence.**

The aim of this thesis was to test the contribution of several factors (G6PD deficiency, mitochondrial retention into mature RBCs and activity of several non-selective

cationic ion channels) on the rheological and senescence properties of RBCs from SCD patients.

In the Study n°1, we tested the impact of the coinheritance of G6PD deficiency with SCD on the RBC rheological properties, RBC senescence markers, hematological parameters, and clinical manifestations. Given that the G6PD deficiency should make RBCs much more sensitive to oxidative stress, the coinheritance of the G6PD deficiency and SCD should result in advanced RBC senescence and greater RBC rheological alterations compared to patients without G6PD deficiency.

In the Studies n°2 and 3 we investigated the associations between mitochondria retention into mature RBCs and RBC rheological and senescence properties in SCA and sickle cell HbSC disease. In addition, we tested the functionality of these residual mitochondria in the study 2 (in SCA). Even in physiological conditions, mitochondria are responsible for the production of ROS, which could promote early senescence and hemolysis of sickle RBCs.

In the Studies n°4 and 5 we studied the role of Piezo1 and TRPV2 on RBC calcium accumulation and RBC rheology in SCD. Both are cation ion channels responsible for the entry of Ca^{2+} into RBCs. We investigated whether their activation was accompanied by membrane hyperpolarization, sickling and advanced senescence.

The materials and methods used for the four studies are reported below, and a brief introduction has been added to introduce each study. The published and submitted articles have been added to the manuscript.

6. Materials and Methods

6.1 Patients' inclusions

For the **Study n°1**, seventy-one patients with homozygous sickle cell disease (SCA, HbSS), no recent blood transfusion or acute complication in the 3 months before the blood collection participated to the study, after given written informed consent. All patients were regularly followed at the Hospital of Lyon (France). Medical records were collected by two physicians to identify patients who had clinical complications in the four years preceding the study.

For the **Study n°2**, sixty-one SCA patients with HbSS genotype, adults, and children at steady state and non-transfused, and 10 healthy donors participated to the study. All patients were regularly followed at the Hospital of Lyon (France) and gave written informed consent.

For the **study n°3**, sixteen HbSC patients, regularly followed at the Hospital of Lyon (France), adults and children at steady state, non-transfused, without clinical complications in the last 3 months before blood collection, and 16 healthy donors participated to this study.

For the **Study n°4**, Patients (HbSS genotype) from Hospices Civils de Lyon and Institut d'Hématologie et d'Oncologie Pédiatrique de Lyon, France, and from the Hematology Center, University of Campinas, Brazil were recruited, with informed consent. Healthy controls participated in the study after giving written informed consent. All subjects

were in clinical steady-state at the time of the study, without experiencing any vaso-occlusive crisis or other acute medical complication within the last 2 months, and without any blood transfusions for at least 3 months before inclusion.

For the **Study n° 5**, ten HbSS patients regularly followed at the Hospital of Lyon, and 4 healthy donors participated to the study. The HbSS patients were regularly followed at the Hospital of Lyon (France), adults and children at steady state, non-transfused, without clinical complications in the last 3 months before blood collection.

6.2 Isotonic red cell deformability

The LORRCA Ektacytometer (Laser Optical Rotational Red Cell Analyzer, RR Mechatronics) was used to measure RBC deformability in an isotonic medium in all the research projects.

The ektacytometry measurement is performed at 37°C in a glass Couette system, which consists of two separate concentric cylinders with a narrow gap between each other. The blood sample is placed in the gap between the two cylinders to be analyzed³¹⁷. During the measurement, the inner cylinder (called bob) remains static, while the external one (cup) moves in rotation (Figure 37 A), creating the following shear stresses: 0,3 Pa; 0,53 Pa; 0,95 Pa; 1,69 Pa; 3 Pa; 5,33 Pa; 9,49 Pa; 16,87 Pa and 30 Pa (Figure 31 C).

For the RBC deformability measurement, 10 μL of blood from sickle cell patients (HbSS and HbSC) or healthy donors (AA) were resuspended in 1 mL of polyvinylpyrrolidone (PVP), a high-viscous medium which has a greater viscosity than the intracellular viscosity of RBCs and which allows the RBCs to deform in the direction of the flow with an elliptical shape²⁸. A 670 nm red laser passes through the suspension and projects the resulting diffraction pattern onto a screen, which is analyzed using software provided by the manufacturer. From the diffraction spectrum of each blood sample, the software calculates the Elongation Index (EI) at each shear stress, corresponding to the long and short axis of the ellipse: $EI = (\text{length} - \text{width})/(\text{length} + \text{width})$ (Figure 37 B).

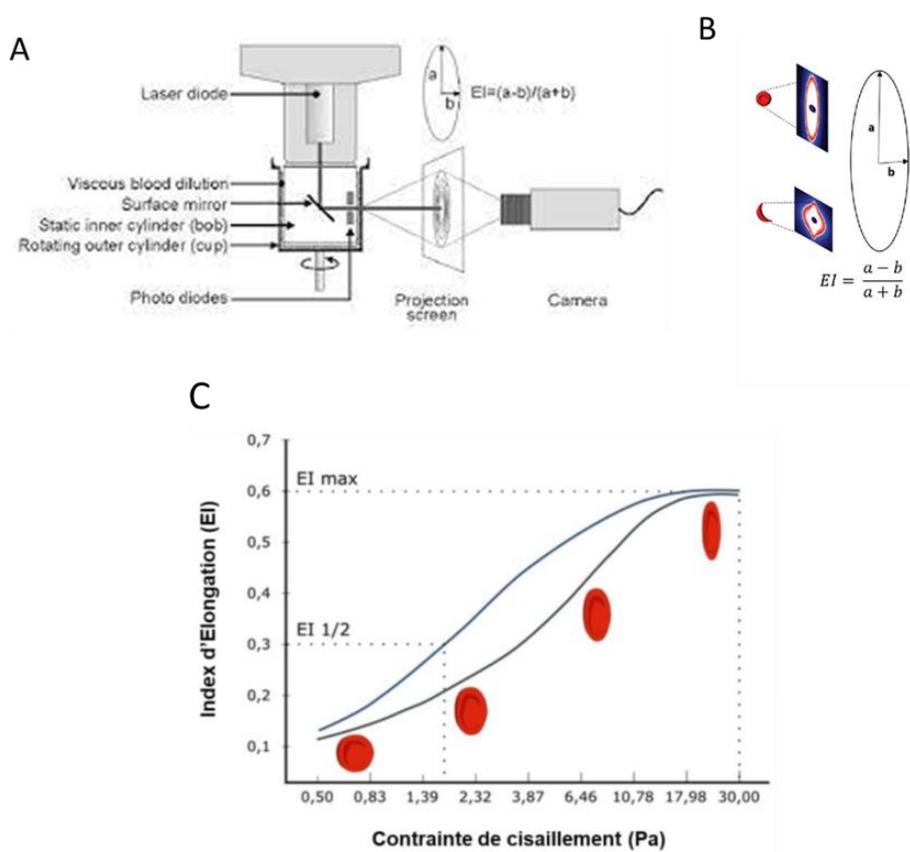


Figure 37: LORRCA system and Representation of RBC deformability measurement (image A), diffraction pattern, and Elongation Index (EI) calculation (image B). Deformability curve and Elongation index depending on shear stress (Pa) (Image C). From Sadaf et al. 2021³¹⁸.

6.3 Red cell deformability over an oxygen gradient

The Oxygenscan protocol from the LORRCA Ektacytometer allows the measurement of RBCs deformability at a given shear stress, during oxygen depletion and re-oxygenation³¹⁹. The Oxygenscan measures the relative oxygen pressure at the critical point at which RBCs start to be rigid and start to sickle, called the “Point of Sickling” (PoS)³¹⁸. The PoS is the most characteristic parameter of this measurement.

For the experiments requiring Oxygenscan measurements, 50 μ L of blood from SCD patients were mixed in 5mL of high viscous (30pC) Oxy-iso PVP medium. The suspension was sheared at 30 Pa at 37°C into the glass Couette system of the Ektacytometer¹⁸⁵. The oxygen partial pressure (Po₂) was gradually decreased from 160 mmHg to 20 mmHg through the injection of nitrogen gas (N₂) in the cell suspension, and then it was re-increased to normoxic values. A 670 nm red laser passes through the suspension and projects the resulting diffraction pattern onto a screen, which was analyzed by the computer. The Oxygenscan readout parameters are:

- Elmax, which represents the RBCs deformability at normoxia;
- Eimin, which represents the deformability upon deoxygenation and at the lowest PO₂.

- The point of sickling (PoS), the PO_2 at which RBCs start to sickle and at which a >5% decrease in EI, compared to Eimax, is observed during deoxygenation (Figure 38).

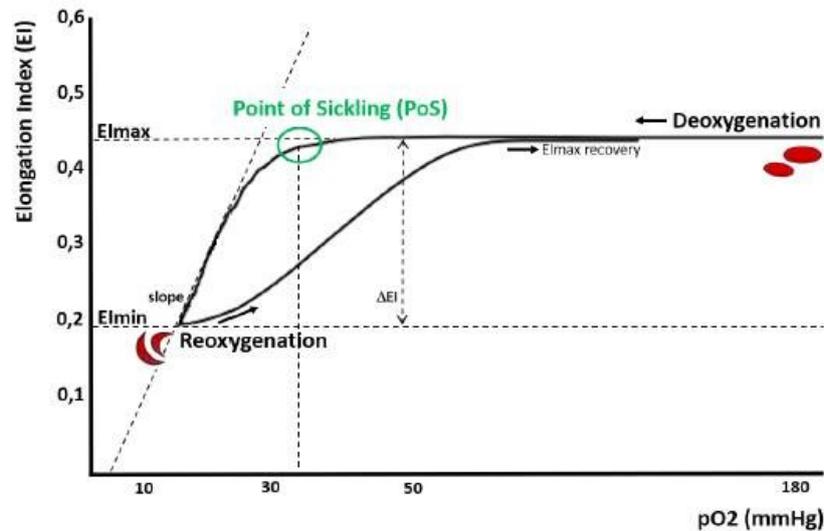


Figure 38. Representative Oxygen scan curve of sickle RBCs. The RBCs suspension is subjected to shear stress and deoxygenation until the reach of the point of sickling (PoS). The Eimax and Eimin showed in the graph indicate the maximal deformability in normoxia and hypoxia conditions. From <https://lorrca.com/wikilorcca/oxygenscan-test/>.

6.4 Flow Cytometry analysis

Flow cytometry is a technology used to rapidly characterize cells and particles in solution by measuring fluorescence, size, and internal complexity or granularity of the cell. This technique is widely used in multiple disciplines such as molecular biology, immunology, and medical monitoring³²⁰. The main advantage of flow cytometry is that it allows the simultaneous characterization of a mixed population of cells, the quantification, and the statistical analysis based on the analytical parameters. In flow

cytometry, cells or particles that are labelled with specific antibodies or probes and resuspended in buffer solution, flow one by one in a capillary system in front of one or more different lasers with different wavelengths. When cells are under the lasers, the stained cell component or markers are excited by the laser and emit light at a longer wavelength than the light source. The light signals are read by detectors, and they are converted into electronic signals and analyzed by a computer. Then, different cell populations can be analyzed according to the:

- The Forward Scatter (FSC), a light signal which is proportional to cell size: the bigger the cell, the more light is scattered, and the higher the detected signal;
- The Side Scatter (SSC), a signal which is proportional to cell complexity; the more organelles/bits inside the cytoplasm, the more light is scattered, and the higher the detected signal.
- The Fluorescence is the signal released after the excitation of the fluorochromes by the lasers. Fluorochromes are molecules linked to antibodies or probes, which can stain specific markers on the RBCs membrane or into the cytoplasm and that generate fluorescent light signals used to analyze the sample.

Several RBC senescence markers, as well as mitochondria retention in sickle RBCs, were analyzed by flow cytometry. The percentages of RBCs exposing PS on the outer membrane were determined using the Annexin-V coupled with a fluorochrome²⁸.

Annexin-V is a protein with a high affinity for PS and which is widely used to detect PS on the surface of RBCs³²¹.

CD47 expression levels at the surface of RBCs were determined using an antibody coupled to a fluorochrome and recognizing the anti-phagocytic antigen CD47.

The intracellular levels of ROS were assessed using the probe 2', and 7'-Dichlorodihydrofluorescein diacetate (H2DCFDA), a probe that can diffuse into the cells and once in the cytoplasm is cleaved by intracellular esterase to 2',7'-Dichlorodihydrofluorescein (H2DCF). Then, when it reacts with intracellular ROS, the H2DCF probe is oxidized and forms a highly fluorescent molecule (2',7'-Dichlorofluorescein, DCF) which can be detected by flow cytometry in the FITC channel³²².

The percentages of sickle RBCs retaining mitochondria were analyzed doing double staining with the anti-CD71 antibody and the Mitotracker deep red probe. The anti-CD71 antibody recognizes the transferrin receptor (CD71) on the reticulocyte's membrane, while the Mitotracker is a mitochondrial potential-dependent dye which diffuses into the mitochondrial membrane and stains active mitochondria into the cells^{88,87}. Doing the double staining, it was possible to discriminate reticulocytes and mature RBC populations retaining mitochondria.

The intracellular Ca²⁺ levels were assessed using another probe, the Fluo-3. As the DCFDA, the Fluo-3 can diffuse into the RBCs and once into the cytoplasm, it is cleaved by intracellular esterases²⁸. When Ca²⁺ ions are present in the cells, the probe reacts

with them forming a fluorescent complex. The fluorescence can be read by the lasers of a flow cytometer, and it can be converted into a positive signal.

Blood samples were centrifuged for 5 min at 1000g and RT. The plasma and buffy coat were removed, and the RBCs pellet was washed twice in PBS 1X buffer and resuspended at 0.2% of hematocrit in the appropriate staining buffer, according to the tested conditions. For the analysis of PS exposure, the Annexin-V staining buffer (Miltenyi Biotech) was used, while for the other senescence markers (ROS, CD47, Ca²⁺, mitotracker, and CD71) PBS 1X buffer was used as the staining buffer. After staining (20 min at room temperature in the dark), samples were washed with PBS 1X and resuspended in PBS 1X or in the appropriate staining buffer. 100µl of the cell suspension was analyzed by Flow cytometry (MacsQuant 16. 100.000 events were recorded and analyzed by FlowLogic software to evaluate senescence the Mean Fluorescence Intensity (MFI) of the different senescence markers and percentages of RBCs retaining mitochondria.

6.5 ImageStream

The ImageStream system, also known as Imaging Cytometry, is a tool for multiparameter cell analysis in flow in real-time, which combines the features of flow cytometry and fluorescence microscopy with a specific methodology for image analysis. Like flow cytometry, ImageStream enables statistical analysis of a cells population based on their fluorescence characteristics. Additionally, using obtained

cellular images that integrate numerous morphometric and photometric characteristics of the analyzed cells, ImageStream enables extensive morphometric cellular analysis. The ImageStream system analyzes cells in suspension, much like flow cytometry³²³. Samples are introduced into a fluidic system that uses a 488 nm solid-state laser and a brightfield light source to illuminate cells hydrodynamically concentrated into a core stream. Then, a microscope objective collects transmitted, scattered, and emitted light. Fluorescence light is then divided into six pictures with specific wavelength ranges by a stack of dichroic filters. The procedure of collecting multispectral data and spectral decomposition into six pictures enables the simultaneous detection of numerous fluorescence pictures in brightfield, darkfield, and other modes (multimode imaging), as well as the quantification of signals from overlapping parts of the examined cell separately (Figure 39).

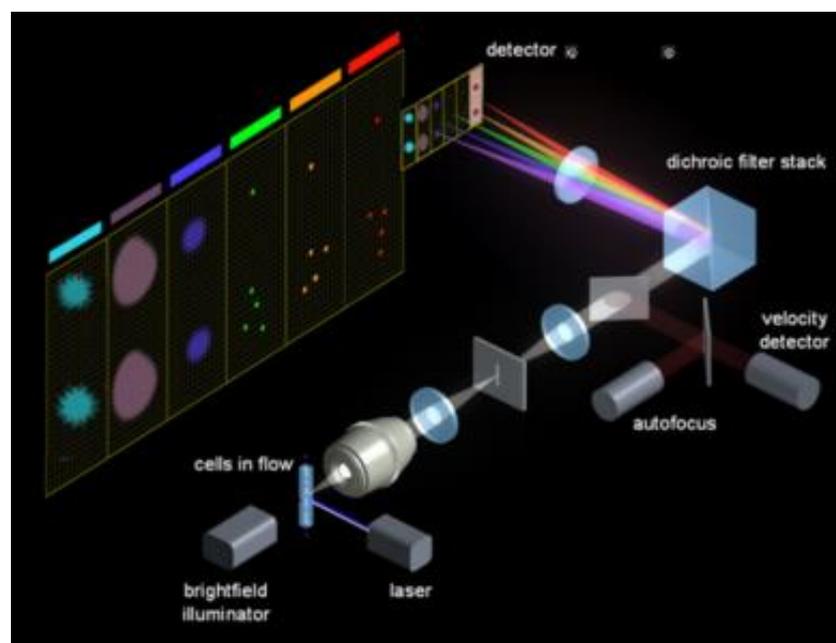


Figure 39. Imagestream platform overview. Cells in laminar flow are collected and illuminated by a 488 laser and red brightfield illumination light. Fluorescent transmitted and scattered lights collected by objective were split into multi-spectral bands by a dichroic filter

stack and the six wavelengths are collected by detectors. From <https://imm.medicina.ulisboa.pt/facility/flowcytometry/doku.php?id=imagestreamx>

ImageStream system was used for the staining and imaging of mitochondria within RBCs from SCA patients and healthy donors.

RBCs were incubated in 100 μ L of prediluted Mitotracker Red CMXRos Dye (Invitrogen, M46752) (1:200 dilution), for 30 min in the dark at RT, to label active mitochondria. After incubation, samples were washed twice in HEPES buffer, the supernatant was removed, and the cells were fixed with 50 μ L of 0.2% glutaraldehyde for 20 minutes. After fixation, RBCs were washed twice with HEPES buffer at 600g for 2 minutes at RT. Images were recorded with Image Stream (Amnis, MK II) and results were analyzed using IDEAS software version 6.2.

6.6 Luminescence-based assay

The luminescence-based assay was used for the evaluation of intracellular oxidative stress, in particular to measure the intracellular levels of GSH, GSSG²⁸⁷. In general, luminescence-based assays are assays in which a luminescent signal, in the form of light or protons, is generated via a chemical or biochemical reaction and it is measured using a plate reader.

Reduced and total (GSSG+GSH) intracellular glutathione was measured using the luminescence-based assay GSH-Glo glutathione Assay (Promega, V6912). Briefly, RBCs

pellets were mixed by inversion in GSH-Glo Reaction buffer (1:5 dilution) to facilitate lysis and centrifuged at 10,000 g for 15 min at 4°C. The supernatant was collected and diluted (1:25 dilution) in deionized water; the reducing agent TCEP was added (30 min in the dark at Room temperature, RT) to reduce the oxidized GSSG and measure the total GSH. 100µl of GSH-Glo reagent (containing Luciferin NT substrate and glutathione S transferase) were added and the samples were incubated for 30 min in the dark at RT. The luminescence was measured using the Synergy Microplate reader. Total GSH corresponds to the measure of the plate containing the reducing agent TCEP. GSSG was calculated subtracting the concentration of GSH (GSH-Glo plate) from the total GSH (obtained with TCEP). The ratio GSH/GSSG was obtained by dividing the GSH concentration by the GSSG concentration³²⁴.

6.7 Manual Patch Clamp

Manual Patch-Clamp is an electrophysiological technique that measures ion currents generated in a single cell upon mechanical or chemical stimulation³²⁵. A glass micropipette containing a microelectrode forms a high resistance seal with the RBCs membrane, “patching” a small part of the membrane containing the ion channel of interest. While the microelectrode is sealed to the cell membrane, this small patch can be ruptured by applying a negative pressure giving the electrode electrical access to the whole cell. Voltage is then applied, forming a voltage clamp, and membrane current is measured (Figure 40). A voltage or current change within the cell

membrane can be altered by applying different drugs or solutions to open or block channels and evaluate the change of currents and membrane hyperpolarization.

Patch clamp experiments were performed in the following solutions: pipette solution (in mM): 140 KCl, 2 MgCl₂, 10 HEPES; pH adjusted to 7.40 with KOH. Bath solution (in mM): 140 NaCl, 2 MgCl₂, 2 CaCl₂, 10 Glucose, 10 HEPES; pH adjusted to 7.40 with NaOH.

To investigate the activation of the TRPV2 channel, we used the Δ^9 THC-ethanol solution (Sigma Aldrich, Saint Louis, MO, USA) or Δ^9 THC-methanol solution (LGC GmbH, Wiesel, Germany). The designed final concentration for each experiment is shown in the detailed method described in the paper. To investigate Gardos Channel interaction with TRPV2 activation, Charybdotoxin (Alomone Labs, Jerusalem, Israel) used as Gardos channel inhibitor, was diluted to a final concentration of 100 Nm.

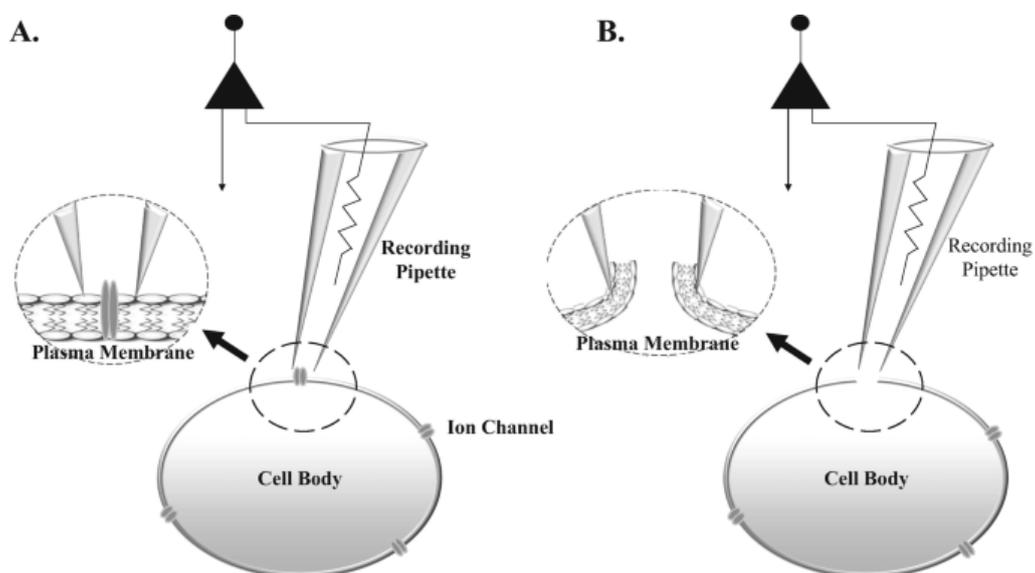


Figure 40. Cell patch clamp technique. A negative pressure is applied to the pipette, which draws the cell membrane into contact with the pipette tip (Figure A). This creates the seal

between the membrane and the pipette, characteristic of a patch clamp recording (Figure B). Image from Leyrer-Jacksin et al., 2019³²⁶.

6.8 Membrane Potential Measurements

The measurement of the average membrane potential variation of RBCs is possible using the MBE (Macey, Bennekou, Egée) method⁴³. The membrane potential can be determined indirectly by measuring the extracellular pH change of RBCs suspension. Two electrodes placed in the RBCs suspension record the pH changes and the signal is converted into a curve of depolarization on a screen.

The ionophore Carbonyl cyanide m-chlorophenyl hydrazone (CCCP, a weak acid) is added in the RBCs suspension, acting as a proton transporter across the cell membrane until an equal distribution of H⁺ is reached between the two sides of the cell. Once the cells are injected into the protonophore-containing solution (CCCP), the buffering capacity of hemoglobin allows a stable measurement of the extracellular pH. It is considered that when the extracellular pH is stabilized in the absence of any other stimulation, the cell is at equilibrium. The pH and the membrane potential are calculated with the following equation:

$$V_M = -61.5(pH_i - pH_o)$$

Then, drugs and channel blockers can be added to the RBCs suspension, causing the hyperpolarization of the membrane. When the ion distribution is altered causing hyperpolarization, the ion pumps act to restore the resting membrane potential through the repolarization of the cell membrane (Figure 41).

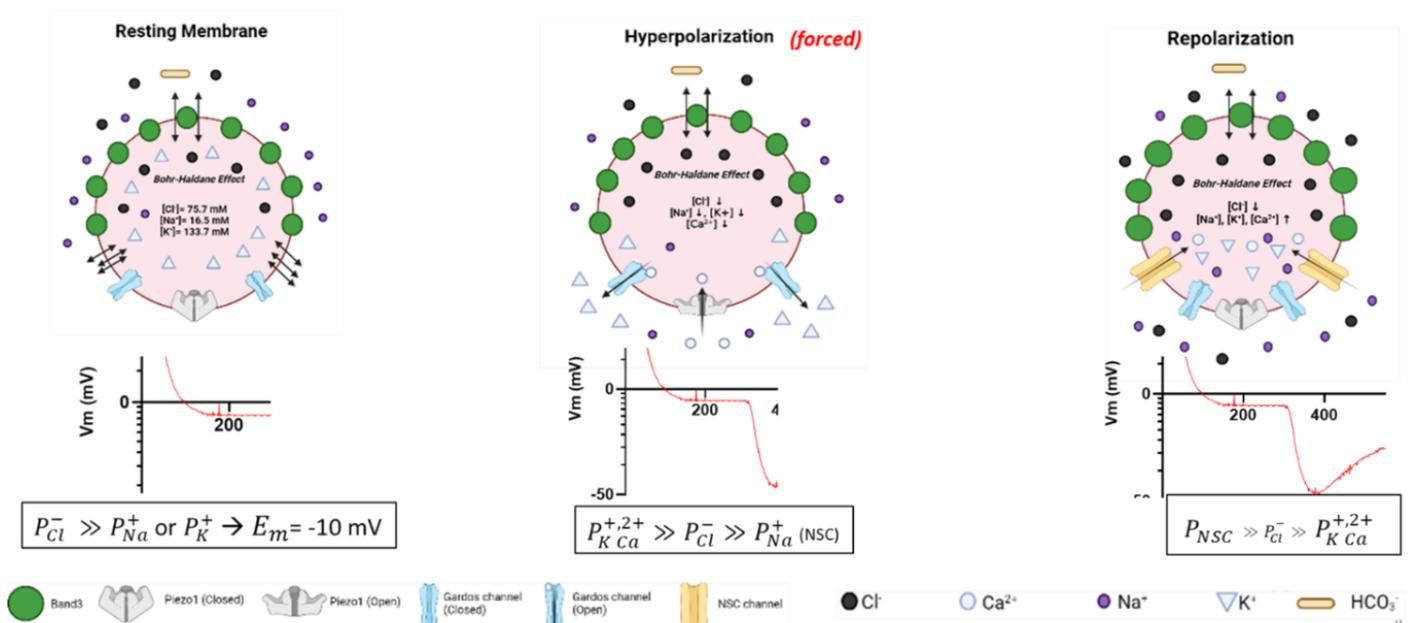


Figure 41. Membrane potential, protein channels, and ions distribution. The intra and extracellular ions distribution at resting membrane, during hyperpolarization and repolarization of RBC. Edited by Aline Hatem, not published.

Blood samples were centrifuged at 2500 g to discard the plasma and the buffy coat, then they were washed three times with Ringer solution (154mM NaCl and 2mM KCl.). Then RBCs were packed at 13,000 g for 2 minutes to reach 99.99% of hematocrit. Washed cells were conserved on ice until used for experiments.

CCCP ionophore (Sigma-Aldrich, Saint Louis, MO, USA) in a final concentration of 20 μM was added in the experimental solution at 37°C, to increase the hydrogen permeability of the membranes.

Extracellular pH was measured using a G200 pH electrode (Radiometer, Copenhagen, Denmark) coupled to a Red Rod 200 reference electrode (Radiometer) and a PHM210 pHmeter (Radiometer). Sampling and acquisition were done with an electrode amplifier (EA-BTA, Vernier, USA) at a rate of 1 measurement per second connected to an AD LABQUEST Mini interface (Vernier, USA) with a resolution of 0.01 pH unit. The data were visualized and analyzed with the Logger Lite software (Vernier, France). At the end of each experiment, Triton X-100 detergent (1% in 3M NaCl) was added, causing total cell lysis and a resulting solution that attains the intracellular pH.

In the study of TRPV2 activation, red blood cells were tested with $\Delta^9\text{THC}$ at two different concentrations: 30 and 80 μM and 230s of recording. To end the experiments, a lysis solution was added. To test Gardos channel contribution, Charybdotoxin was added at 200s to pre-block its activation. The deoxygenated conditions were performed in deoxygenated Ringer solution, perfused with Nitrogen (N_2) gas before and during the experiment (oxygen concentration of 0.8 mg/L).

Recordings were made using the Logger Lite (Vernier; Millikan Way, Beaverton, OR 97005 France) program at an acquisition rate of 1 point/s. Data were treated using Excel (Microsoft; Redmond- Washington, USA) and MATLAB R2022a.

6.9 High resolution respirometry

High resolution respirometry test was performed using the Oroboros O2K, a unique technology which allows the measurement of oxygen consumption of biological or biochemical samples. Several activators or inhibitors of the different mitochondrial complexes (such as Oligomycin, FCCP and Antimycin A) are added in the chambers containing the cell suspensions, to increase, decrease and then evaluate the oxygen consumption (Figure 42).

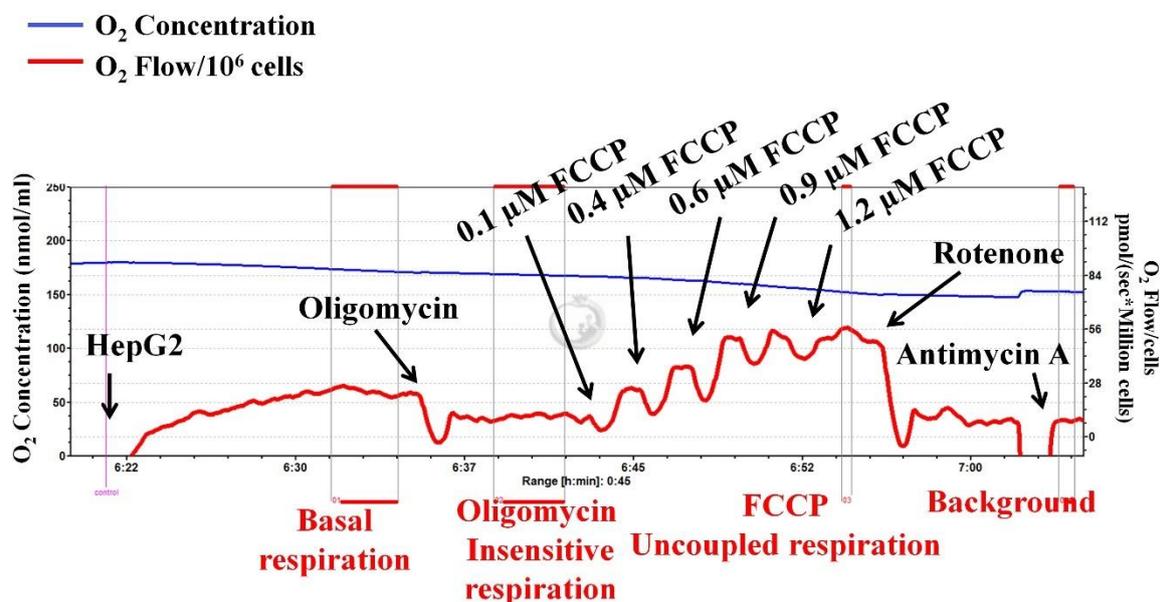


Figure 42. Example of coupled and uncoupled respiration of intact cells using high-resolution respirometry. Basal oxygen consumption measured in the presence of oligomycin (oligomycin-insensitive respiration) and addition of FCCP. Then, respiration is inhibited with the addition of antimycin A, leaving the remaining background respiration (non-mitochondrial respiration). Oxygen consumption is expressed as pmol/(sec x number of cells). From Djafarzadeh et al. 2017³²⁷.

We used a high-resolution respirometry protocol for intact blood cells adapted from Sjövall et al.³²⁸ and Stier et al.³²⁹ The respirometry test was performed on mature sickle RBCs isolated through Percoll density gradient¹⁸. Immediately before the start

of the mitochondrial measurements, Percoll-isolated RBCs stored for < 1 hours in PBS at 4°C were centrifuged at 800 *g* for 2 min to pellet the cells. The supernatant was removed and 100µL of packed cells was re-suspended in 1mL of respiratory buffer MiR05 (0.5 mM EGTA, 3 mM MgCl₂, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH₂PO₄, 20 mM Hepes, 110 mM sucrose, free fatty acid bovine serum albumin (1 g/L), pH 7.1) pre-warmed at 37°C, and transferred in the respirometry chamber of one Oxygraph-2k high-resolution respirometer (Oroboros Instruments, Innsbruck, Austria) set at 37°C and containing 1mL of Mir05. Endogenous O₂ consumption was then recorded after approximately 10 min of stabilization. ATP-dependent O₂ consumption was inhibited by adding oligomycin (2.5µM final), an inhibitor of ATP synthase. The mitochondrial uncoupler FCCP (carbonyl cyanide-p-trifluoro-methoxyphenyl-hydrazine) was then titrated in 0.05 µM steps until the maximal uncoupled O₂ consumption was reached. Finally, mitochondrial O₂ consumption was fully inhibited by adding antimycin A (2.5 µM). Consumption of O₂ for each step was then extracted using DatLab version 7.0 software. RBCs from eight SCA patients and five healthy controls after the Percoll separation were used for these experiments.

6.10 Red blood cell microfluidic adhesion assay

RBC adhesion and rolling on laminin-coated microchannels (400 µm-wide microchannels, Cellix Ltd, Ireland) were observed using a microfluidic platform (Cellix Ltd, Ireland). Biochip channels were coated with 4 µg/mL human laminin (LN, from

human placenta, Sigma Aldrich) for 2 h at RT. The LN ligand concentration was chosen based on standardization assays, employing ligand concentrations of 2-10 $\mu\text{g/ml}$ (data not shown). To test whether Piezo1 activation could modulate sickle RBCs adhesive interaction to laminin, sickle RBCs were preincubated with Yoda1 (0.1 – 10 μM 30 min). After the preincubation, their rolling along and adhesion to Laminin (LN) was evaluated under shear stress conditions using a microfluidic flow assay (3 min, 37°C, 0.25 dyn/cm^2).

Non-specific binding sites on channels were then blocked with 1% (w/v) BSA (30 min, RT) and chips were stored in a humidified chamber for use in assays within 1 hour. After treatments, cell suspensions were loaded sequentially, onto microchannel entry wells, and a shear stress of 0.25 dyn/cm^2 applied for >3 min (ExiGo Pump Pulse-Free Microfluidic Syringe Pump). Images for each channel, in 3 fields, were acquired (1 per second) for video manufacture using a Zeiss microscope (X40 lens) and Exi Blue Cooled camera Camera (Qimaging, Surrey, Canada). Numbers of cells rolling along the chip (defined as a significant decrease in cell velocity through the channel, to 50% or less) and adhered to the chip were evaluated in 3 fields at 180 seconds.

7. Studies

7.1 Study n°1

“Does G6PD deficiency cause further damage to red blood cells of patients with sickle cell anaemia?”

Esperti S, Boisson C, Robert M, Nader E, Cibiel A, Renoux C, Horrand F, Gauthier A, Poutrel S, Joly P, Connes P. Does G6PD deficiency cause further damage to red blood cells of patients with sickle cell anaemia? *Br J Haematol.* 2022 Oct;199(2):289-291. doi: 10.1111/bjh.18417. Epub 2022 Aug 16. PMID: 35973746.

Oxidative stress is a hallmark of SCD, and several mechanisms contribute to the high levels of ROS in sickle cell patients, including the higher autoxidation of sickle hemoglobin, the chronic proinflammatory state, the characteristic recurrent ischemia reperfusion injury²⁴⁵. Accumulation of intracellular ROS affects RBCs membrane structure and function, compromising the membrane integrity and decreasing the RBCs deformability³¹⁶, promoting PS exposure and release of microvesicles³³⁰. The increased production of ROS results in increased hemolysis, endothelial dysfunction and increased hemolytic complications³³¹. As previously introduced, increased baseline of oxidative stress is the hallmark of another inherited disorder, the G6PD deficiency, another condition which in some cases could be present simultaneously with SCD.

The aim of this study was to investigate the effect of the coinheritance of Sickle cell disease and Glucose-6-phosphate dehydrogenase deficiency on the rheological properties and RBCs senescence markers, hematological parameters, and clinical manifestations. We analyzed fresh blood samples from seventy-one patients with homozygous form of SCA without recent blood transfusions, at steady state and without clinical complications. After an enzymatic screening for G6PD deficiency, we found 10 patients having both SCA and G6PD deficiency and compared biological/clinical parameters to SCA patients without G6PD deficiency.

As expected, we observed that the group with SCA and G6PD deficiency had increased levels of intracellular oxidative stress. However, no other difference in RBC

senescence markers, rheologic parameters or clinical outcomes was observed between the two groups of patients. Our study demonstrated that the coinheritance of SCA and G6PD deficiency does not cause further damages to sickle RBCs. However, the impact of G6PD deficiency in SCA patients is still debated, and although we did not observe other differences in SCA patients having also G6PD deficiency, it is recommended to them to not consume oxidative substances which could worsen their hematological and clinical phenotype.

Does G6PD deficiency cause further damage to red blood cells of patients with sickle cell anaemia?

Glucose-6-dehydrogenase (G6PD) deficiency is the most common enzyme deficiency in the world,¹ which leads to a lower level of reduced glutathione, an antioxidant, in red blood cells (RBCs). Most of the time, those who are affected have no symptoms. However, they should avoid specific triggers that may promote oxidative stress such as fava beans, that may fragilize RBCs and cause haemolysis. G6PD deficiency has been proposed to be a modulator of certain complications in sickle cell anaemia (SCA), such as stroke² and vaso-occlusive crises,³ but others found no major role of G6PD deficiency in the clinical manifestations and haematological phenotype of SCA.⁴⁻⁶ The present study investigated the effects of G6PD deficiency on RBC deformability, RBC senescence markers, haematological parameters and clinical severity in SCA. We hypothesized that RBC oxidative stress would be greater in SCA patients with G6PD deficiency than those without, and this would be accompanied by greater alterations in RBC rheology.

Seventy-one patients with homozygous sickle cell disease (SCA, HbSS), no recent blood transfusion or acute complication participated in the present study after giving written informed consent. Medical records were reviewed by two physicians to identify patients who had acute chest syndrome (ACS) or vaso-occlusive crises (VOC) in the four years preceding the study, as well as a diagnosis of osteonecrosis, glomerulopathy, retinopathy and leg ulcers. A patient was considered as having frequent VOC when the number of VOC per year was greater or equal to two. This study was performed in accordance with the Declaration of Helsinki and approved by the HCL Ethics committee (L14-127). Screening for G6PD deficiency in SCA patients was first done at the enzyme level in all patients. Both G6PD and pyruvate kinase (PK) activities were measured and the ratio G6PD/PK calculated. A ratio comprised between 0.5 and 1.5 indicates no deficiency. For hemizygotes or homozygotes, the ratio is very low (<0.10). When the ratio was lower than 0.5, high resolution melting (HRM) analysis was performed focusing on the Med and A(-) variants⁷ to detect a possible heterozygous carrier state.

Haematological parameters were determined with a haematology analyser (Advia, Siemens, Rungis, France). Ektacytometry was carried out with the Laser-assisted optical rotational red cell analyser (Lorrca, RR-Mechatronics) with the oxygenscan module to measure RBC deformability

(Elongation Index; EI) over an oxygen gradient and at 30 Pa, as previously described.⁸ Several parameters were derived: (1) EI_{max} (normoxic RBC deformability), (2) EI_{min} (the lowest RBC deformability reached during deoxygenation) and (3) PoS (the pO₂ at which RBC deformability decreases below 95% of EI_{max} during deoxygenation), which reflects the pO₂ at which RBCs start to sickle during deoxygenation.

Phosphatidylserine (PS) exposure at the outer membrane leaflet of RBCs and CD47 were assessed using Annexin V-PE (Miltenyi, 130-118-363) and anti-CD47-PE antibody (Miltenyi 130-101-348), respectively.⁹ Intracellular RBC reactive oxygen species (ROS) were determined using 2',7'-dichlorofluorescein diacetate (DCFDA, Sigma-Aldrich, Saint-Quentin-Fallavier, France).⁹ DCFDA is a non-specific probe that allows the determination of the degree of overall oxidative stress in the cells by reacting with various reactive oxygen species. Despite the fact that nitric oxide may oxidize DCFDA, this probe seems sensitive enough to detect intracellular oxidative stress levels in RBCs in a pathophysiological context and for *in vitro* pharmacological modulation assessments.⁹

Ten patients had G6PD deficiency (six homozygotes, four hemizygotes), all A(-) variants. Age was not different between the two groups (23.7 ± 16.8 vs 24.9 ± 13.8 years in patients with and without G6PD deficiency, respectively). Biological results (Table 1) showed greater intracellular ROS content and higher percentage of positive RBCs for ROS in SCA patients with G6PD deficiency. RBC ROS level was also analysed in a group of healthy subjects (36.9 ± 7.8 years): positive RBCs = 55.0% ± 24.3%; median fluorescence intensity (MFI) = 0.57 ± 0.14. Both parameters were below the values found in the two SCA groups. The other markers were not different between the two groups. Table 2 shows no difference in the clinical parameters between the two groups.

Bouguerra *et al.*¹⁰ previously showed higher numbers of PS-exposing RBCs and higher ROS level in the RBCs from non-SCA G6PD-deficient patients than from healthy volunteers. Indeed, it is not surprising that greater intracellular ROS levels were observed in SCA patients with G6PD deficiency than in those without in the present study. However, no difference was observed between the two groups for RBC senescence markers (percentage of RBCs exposing PS and CD47 expression), RBC deformability in both normoxia and hypoxia, the propensity of sickling (i.e., PoS), the level

TABLE 1 RBC senescence markers, RBC deformability and haematological parameters in SCA patients without G6PD deficiency and with G6PD deficiency

	No G6PD deficiency	G6PD deficiency
ROS (MFI)	1.1 ± 0.5	2.0 ± 2.0**
ROS+ RBCs (%)	84 ± 18	95 ± 6*
CD47 (MFI)	6.6 ± 1.6	6.3 ± 1.5
PS+ RBCs (%)	2.3 ± 1.6	2.3 ± 1.5
EI _{min}	0.08 ± 0.06	0.09 ± 0.06
EI _{max}	0.45 ± 0.07	0.43 ± 0.05
PoS (pO ₂ in mm Hg)	40.4 ± 7.0	39.8 ± 5.0
Hb (g/l)	94 ± 15	93 ± 21
Hct (%)	26.6 ± 4.2	26.5 ± 5.0
MCV (fl)	84 ± 14	81 ± 14
MCHC (g/l)	348 ± 15	340 ± 24
Reticulocytes (%)	14.1 ± 8.7	10.7 ± 6.2
LDH (IU)	431 ± 154	448 ± 269
HbF (%)	14.1 ± 8.7	10.7 ± 6.2
HbS (%)	81.0 ± 13.6	82.9 ± 15.6

Abbreviations: EI_{min}, minimum RBC deformability reached during deoxygenation; EI_{max}, maximum RBC deformability in normoxia; Hb, haemoglobin; HbF, foetal Hb; HbS, haemoglobin S; Hct, haematocrit; IU, international unit; LDH, lactate dehydrogenase; MCHC, mean corpuscular haemoglobin concentration; MCV, mean cell volume; MFI, median fluorescence intensity; PoS, Point of Sickling (pO₂ at which sickling starts during deoxygenation); PS, phosphatidylserine; PS+ RBCs, RBCs positive for PS; RBC, red blood cell; ROS, reactive oxygen species; ROS+ RBCs, RBCs positive for ROS. **p* < 0.05; ***p* < 0.01.

TABLE 2 Clinical complications in SCA patients without G6PD deficiency and with G6PD deficiency

	No G6PD deficiency	G6PD deficiency
Hydroxyurea (hydroxycarbamide) treatment (%)	77%	100%
One α gene deleted (%)	0	9.8
Two α genes deleted (%)	50	42.6
Leg ulcers (%)	6.6	0
Osteonecrosis (%)	27.9	20
Retinopathy (%)	14.8	20
Frequent VOC (%)	14.8	10
ACS (%)	24.6	30
Glomerulopathy (%)	4.9	0

Abbreviations: ACS, acute chest syndrome; VOC, vaso-occlusive crisis.

of anaemia (Hct and Hb) and haemolytic markers. Although some studies reported lower Hb concentrations in SCA patients with G6PD deficiency compared to those without,^{11,12} the majority of previous studies showed no difference in haematological parameters between the two populations.^{13,14} Nevertheless, these results are quite surprising since oxidative stress is known to further reduce RBC deformability in

SCA and fragilize RBC,⁹ as well as promote RBC senescence. The exact reasons for these discrepancies are not clearly understood. Although our study did not show further RBC alterations in G6PD-deficient patients, the higher baseline RBC oxidative stress found in this group justifies patients becoming acquainted with their G6PD genotype in order to avoid consuming oxidative substances that would further enhance oxidative stress and worsen their haematological phenotype.

The clinical impact of G6PD deficiency in SCA is also debated in the literature. Some studies reported an association between cerebral vasculopathy and G6PD deficiency^{2,15} while others did not find such an association.^{5,6} Patients with a chronic transfusion programme were not included in this study and it was thus not possible to test whether G6PD deficiency was associated with brain infarcts or abnormal transcranial Doppler velocity since these patients usually have transfusion therapy. Instead, we tested the associations between G6PD deficiency and other acute and chronic complications. Although the sample size of our G6PD-deficient group was small, we did not detect any association which confirms previous studies.^{5,13,14}

In conclusion, our study did not show any difference in the RBC rheological, haematological and clinical profiles between SCA patients with and without G6PD deficiency despite higher RBC oxidative stress in the former population.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

Data are available upon requests to the corresponding author.

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7.2 Study n°2

Increased retention of functional mitochondria in mature sickle red blood cells is associated with increased sickling tendency, hemolysis and oxidative stress

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Increased oxidative stress is a hallmark of SCD and it plays a significant role in the pathophysiology of the disease and associated complications. Several mechanisms are responsible for the increased oxidative stress in SCD, but the recent discovery of residual mitochondria within sickle RBCs has made these organelles excellent candidates among those responsible for increased oxidative stress. Mitochondria are an important source of reactive oxygen species which could contribute to the oxidative damage in sickle RBCs.

In this study, we analyzed the presence and the functionality of abnormal retained mitochondria within sickle RBCs from SCA patients and we tested the associations with senescence and oxidative stress markers, hematological parameters and rheologic profiles of RBCs. The presence of mitochondria within mature sickle RBCs from 71 SCA patients and 10 healthy donors was assessed by flow cytometry by using a specific probe for mitochondria (Mitotracker deep red) and an anti-reticulocytes antibody (CD71) to discriminate the mitochondria from the two different cell populations. Then, SCA patients were classified into three groups according to the percentage of RBCs retaining mitochondria: low (0-4%), moderate (>4 and <8%), or high level (>8%). We also analyzed the levels of senescence markers (such as Phosphatidylserine exposure, CD47 and Ca²⁺ levels) and oxidative stress markers (intracellular ROS and Glutathione levels) of sickle RBCs as well as the rheological parameters (RBC deformability under normoxia (Elongation Index, Eimax), and the propensity of RBCs to sickle under deoxygenation (Point of Sickling; PoS). Then, the

functionality of residual mitochondria in RBCs from eight SCA patients and 8 healthy donors was measured by high-resolution respirometry assay. The results from high-resolution respirometry assay showed that mitochondria in mature RBCs from the SCA group still have an activity, with about half of the oxygen consumption used for ATP production. Furthermore, patients with higher percentages of RBCs retaining mitochondria had higher levels of PS exposure, lower RBC deformability and increased propensity of RBC to sickle upon deoxygenation, as well as lower hematocrit and increased hemolytic markers. We didn't observe any difference in reduced (GSH) and oxidized (GSSG) Glutathione and in the ratio between the two forms. We also compared mitochondria-positive RBCs to mitochondria-negative RBCs and found increased ROS and Ca^{2+} levels, greater percentages of RBCs positive for PS and lower CD47 expression. These results suggest that the retention of functional mitochondria within sickle RBCs could promote RBC senescence, decrease RBC deformability, and increase their propensity to sickle upon deoxygenation.

Increased retention of functional mitochondria in mature sickle red blood cells is associated with increased sickling tendency, hemolysis and oxidative stress

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Abstract

Abnormal retention of mitochondria in mature red blood cells (RBC) has been recently reported in sickle cell anemia (SCA) but their functionality and their role in the pathophysiology of SCA remain unknown. The presence of mitochondria within RBC was determined by flow cytometry in 61 SCA patients and ten healthy donors. Patients were classified according to the percentage of mature RBC with mitochondria contained in the whole RBC population: low (0–4%), moderate (>4% and <8%), or high level (>8%). RBC rheological, hematological, senescence and oxidative stress markers were compared between the three groups. RBC senescence and oxidative stress markers were also compared between mature RBC containing mitochondria and those without. The functionality of residual mitochondria in sickle RBC was measured by high-resolution respirometry assay and showed detectable mitochondrial oxygen consumption in sickle mature RBC but not in healthy RBC. Increased levels of mitochondrial reactive oxygen species were observed in mature sickle RBC when incubated with Antimycin A *versus* without. In addition, mature RBC retaining mitochondria exhibited greater levels of reactive oxygen species compared to RBC without mitochondria, as well as greater Ca²⁺, lower CD47 and greater phosphatidylserine exposure. Hematocrit and RBC deformability were lower, and the propensity of RBC to sickle under deoxygenation was higher, in the SCA group with a high percentage of mitochondria retention in mature RBC. This study showed the presence of functional mitochondria in mature sickle RBC, which could favor RBC sickling and accelerate RBC senescence, leading to increased cellular fragility and hemolysis.

Introduction

Sickle cell anemia (SCA) is an inherited hemoglobinopathy caused by a single point mutation in the β -globin gene which leads to the synthesis of an abnormal hemoglobin, called hemoglobin S (HbS). When deoxygenated, HbS polymerizes causing a mechanical distortion (i.e., sickling) of

red blood cells (RBC).¹ Sickle RBC are less deformable and more fragile than normal RBC, resulting in chronic anemia² and frequent vaso-occlusive crises.³ Moreover, the accumulation of free hemoglobin and heme in the plasma promotes oxidative stress, inflammation and endothelial dysfunction, which contribute to the pathophysiology of SCA and the development of chronic complications.⁴

Recent studies reported the presence of mitochondria in mature RBC in patients with SCA.⁵⁻⁷ Mitophagy is an important step for the survival of RBC during erythroid maturation.⁸ It has been demonstrated that the abnormal mitochondria retention in sickle RBC would be the result of a deficient mitophagy pathway throughout erythropoiesis.⁵ Jagadeeswaran *et al.*⁹ demonstrated that the use of the lysine-specific demethylase 1A (LSD1) inhibitor (RN-1), and the use of a mitophagy-inducing agent mammalian target of rapamycin (mTOR) inhibitor (sirolimus) increased RBC lifespan in a sickle cell mouse model. Of note, LSD1 inhibitor RN-1 could also improve RBC lifespan through its effects on HbF synthesis, as reported in non-human primates.¹⁰ In another study, it has been reported that SCA patients with a high percentage of mature RBC containing mitochondria exhibited higher levels of reticulocytes and total bilirubin, suggesting increased hemolysis in these patients compared to those with a low percentage of mature RBC with mitochondria.⁵ Indeed, although not formally proved, it has been hypothesized that the retention of mitochondria in mature RBC would favor hemolysis.⁹

It has been proposed that mitochondria retention in mature RBC could cause a shortening of their lifespan because of the accumulation of reactive oxygen species (ROS) generated by metabolically active mitochondria¹¹ that would damage the cell.¹² Promoting mitophagy in sickle cell mice was accompanied by a decrease in RBC ROS content.⁹ However, Martino *et al.*⁵ did not show any difference in the mitochondrial membrane potential using Mitotracker orange between patients with mature SCA RBC containing mitochondria compared to those without. Moreover, transmission electron microscopy experiments revealed that mitochondria in mature RBC were swollen, small and disorganized.⁵ It was concluded that mitochondria in mature RBC in SCA patients are not functional. However, another recent study supports the opposite.⁶ Proteomic, metabolomic and lipidomic analyses of mature RBC containing mitochondria and of mature RBC without mitochondria were performed, and higher levels of mitochondria metabolites were observed in the first population.⁶ Subsequent analyses in one patient with SCA showed detectable levels of oxidative phosphorylation activity and the presence of mitochondrial electron chain components in mature RBC containing mitochondria, supporting the idea that mitochondria would be functional. Indeed, the question of the functionality of mitochondria in mature RBC of SCA patients is still debated, and how mitochondria retention in mature RBC could be responsible for a rise in hemolysis in SCA remains unclear.

The aim of this study was to test the associations between sickling tendency, RBC senescence, oxidative stress, hematological markers and the levels of mature RBC containing mitochondria in SCA patients and to investigate the functionality of these mitochondria.

Methods

More details on the methods are given in the *Online Supplementary Appendix*. Blood samples from 61 SCA patients (HbSS genotype, 29 men and 32 women, at steady state, 23.2±14.2 years, non-transfused) and ten healthy race-matched donors (AA) were collected in EDTA tubes. The study was conducted in accordance with the guidelines set by the Declaration of Helsinki, and all subjects gave informed written consent. The study was approved by the Regional Ethics Committees (L16-47, CPP Sud-Est IV, Hospices Civils de Lyon).

Image Stream (Amnis, MK II) and Mitotracker Red CMXRos Dye (Invitrogen) were used to label active mitochondria. Flow cytometry (MACSQuant 16, Miltenyi), MitoTracker^(®) Deep Red probe (MTKdr, Sigma-aldrich) and anti-CD71 antibody were used to determine the percentages of reticulocytes and mature RBC containing mitochondria. Since no criteria or threshold exist in the literature to decide which patient has high or low mitochondria retention in mature RBC, patients were divided into three groups using terciles according to the percentages of mature RBC containing mitochondria in the whole RBC population: “high”, “moderate” and “low” percentage of RBC containing mitochondria. Triple staining on blood samples from ten SCA patients was performed with anti-CD235a (Miltenyi) and anti-CD41 (Miltenyi) antibodies and Mitotracker deep Red probe, to gate on the RBC⁺/platelet⁻ population.

Blood samples from eight patients and five AA were used for percoll gradient separation. The second layer containing mature RBC was collected and the presence of reticulocytes was assessed by Flow Cytometry by using an anti-CD71 antibody (Miltenyi). Then, we investigated the functionality of mitochondria in mature RBC from SCA patients using a high-resolution respirometry protocol for intact blood cells.^{13,14} Endogenous O₂ consumption was recorded before inhibiting ATP-dependent O₂ consumption with oligomycin (2.5 μM), an inhibitor of ATP synthase. The mitochondrial uncoupler FCCP (carbonyl cyanide-p-trifluoro-methoxyphenyl-hydrazone) was then titrated in 0.05 μM steps until the maximal uncoupled O₂ consumption was reached. Finally, mitochondrial O₂ consumption was fully inhibited by adding Antimycin A (2.5 μM).

Flow cytometry was also used to determine the percentage of RBC exposing phosphatidylserine (PS) (Annexin-V-PE, Miltenyi 130-118-363), the anti-phagocytic CD47 antigen (anti-CD47-PE antibody, Miltenyi) and intracellular ROS levels (2',7'-dichlorofluorescein diacetate [DCFDA], Sigma-Aldrich) of the three groups categorized according to the percentages of mature sickle RBC containing mitochondria. Double staining for mitochondria retention (Mitotracker Deep Red) and i) intracellular Ca²⁺ (Fluo3 AM, ThermoFischer), ii) CD47, iii) PS or iv) intracellular ROS, was performed to compare these parameters between mature

RBC containing mitochondria and those without. Mitochondria superoxide production in sickle RBC was assessed using the MitoSOX Red mitochondria superoxide indicator (Invitrogen, M36008) and flow cytometry, with and without Antimycin A, an inhibitor of the Respiratory Complex III. Reduced (GSH) and total (GSSG+GSH) intracellular glutathione were measured using the luminescence-based assay GSH-Glo glutathione Assay (Promega). Oxygen gradient ektacytometry was performed using the Oxygenscan protocol of the LORRCA Maxis (Mechatronics) to measure RBC deformability in an oxygen gradient.¹⁵¹⁶ The maximum RBC deformability (Elmax) reached in normoxia and the oxygen pressure at which RBC start to sickle (point of sickling [PoS]) were determined.

containing mitochondria from three SCA patients are shown in Figure 1A. Figure 1B shows the lack of mitochondria in RBC from three healthy donors. Flow cytometry analyses (gating strategy in Figure 1C) demonstrated no mitochondria in mature RBC from healthy individuals and highly variable percentages of mature RBC containing mitochondria in patients with SCA (Figure 1D). The mean percentages of mature RBC containing mitochondria in the three SCA groups categorized according to the percentages of mature RBC containing mitochondria in the whole RBC population were: low group 1.40±1.31%, moderate group 6.2±1.02% and high group 13.02±5.84%. The high-resolution respirometry assay showed active oxygen consumption being responsive to specific mitochondrial inhibitors (Figure 2A). Flow cytometry analysis of the isolated RBC fraction obtained after the Percoll separation gradient showed a very low percentage of residual reticulocytes (<0.6%) and platelets (<0.5%), indicating that the respirometry results were mainly attributable to sickle RBC. Specifically, oxygen consumption was observed in mature sickle RBC but not in healthy RBC (Figure 2A, interaction patient status *respiratory rate; *P*<0.001). As expected, respiration was undetectable and unaffected by

Results

Presence of functional mitochondria in mature sickle red blood cells

Image Stream analysis showed the presence of mitochondria in mature RBC isolated from SCA patients, but not in RBC from healthy donors. Representative images of RBC

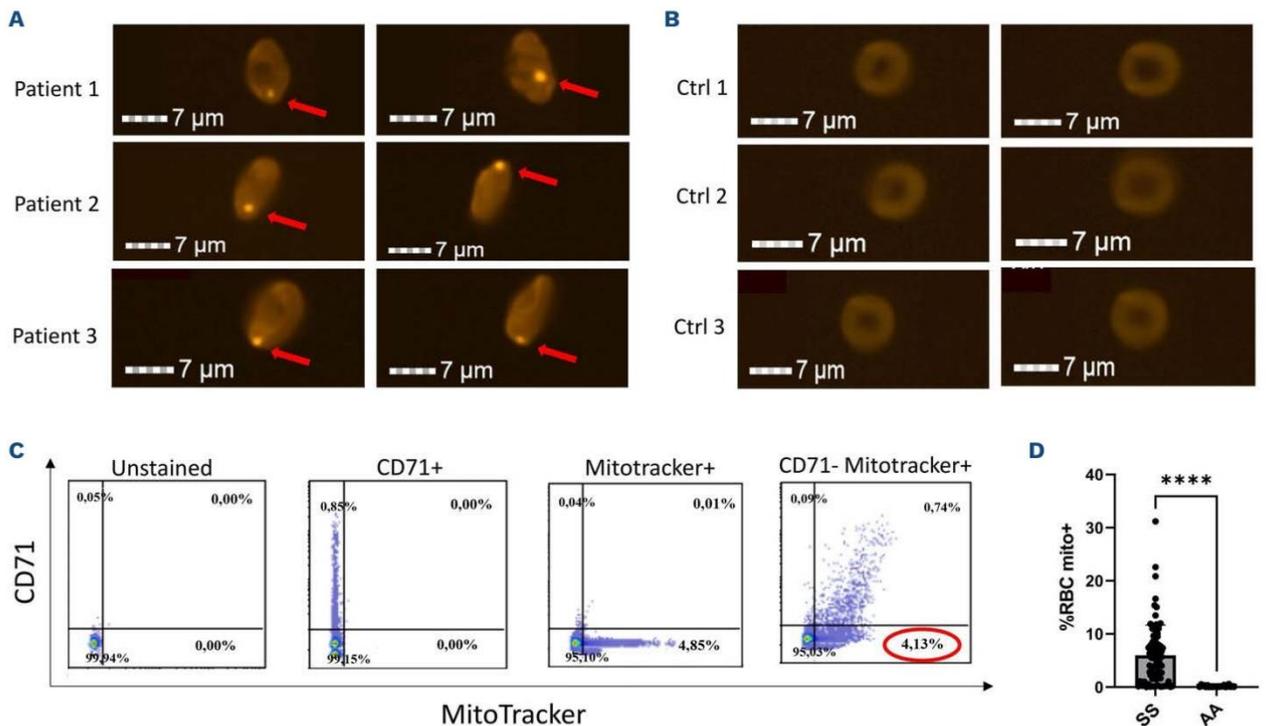


Figure 1. Presence of mitochondria in mature sickle red blood cells. (A) ImageStream and staining with Mitotracker Red CMXRos dye of mitochondria in mature sickle red blood cells (RBC) of three sickle cell disease (SCA) patients. (B) ImageStream and staining with Mitotracker Red CMXRos dye show no mitochondria in RBC from healthy donors. (C) Gating strategy for flow cytometry analysis to discriminate mature sickle RBC retaining mitochondria (CD71+ Mitotracker+). (D) Mean percentages of mature RBC with mitochondria (mito) in 61 SCA (SS) patients and 10 healthy (AA) donors. Significant difference: *****P*<0.0001.

the mitochondrial inhibitors/uncoupler (oligomycin, FCCP, and Antimycin A; all $P>0.24$) in healthy controls. In SCA patients, endogenous respiration was detectable and decreased after adding Oligomycin (Figure 2A; $P<0.001$), an inhibitor of ATP synthase (ATP-inhibited), showing that approximately half of the endogenous oxygen consumption was coupled to ATP synthesis in mature sickle RBC. Following the addition of the uncoupler FCCP thereby reducing the proton force in the inner mitochondrial membrane (i.e., uncoupled O_2 consumption), the cellular respiration of mature sickle RBC increased back to the endogenous level ($P<0.001$). Finally, the use of Antimycin A, an inhibitor of mitochondrial complex III, caused a marked reduction of O_2 consumption in mature sickle RBC (i.e., non-mitochondrial O_2 consumption; $P<0.001$). Superoxide anion accumulation from mitochondria in mature RBC from 14 SCA patients was detected by the MitoSox red probe with and without the use of Antimycin A. The percentages of mature sickle RBC accumulating mitochondrial superoxide anion increased after the incubation with Antimycin A (Figure 2B).

Mitochondria retention in mature sickle red blood cells (RBC) is associated with increased RBC senescence markers and increased propensity of RBC to sickle under deoxygenation

The comparisons of the three groups categorized ac-

ording to the percentages of mature sickle RBC retaining mitochondria contained in the whole RBC population showed no difference in intracellular ROS (Figure 3A), reduced (GSH, Figure 3B), oxidized (GSSG, Figure 3C) glutathione levels and the ratio between the two forms (Figure 3D). No difference in anti-phagocytic CD47 expression was observed between the three groups (Figure 3E). The percentages of RBC with externalized PS were greater in the groups with moderate and high percentages of mature RBC containing mitochondria (Figure 3F). The comparisons between mature sickle RBC with or without mitochondria showed higher Ca^{2+} levels, intracellular ROS, and lower CD47 expression in RBC retaining mitochondria (Figure 4A-C). Figure 4D shows greater percentages of RBC with externalized PS in mature sickle RBC with mitochondria compared to those without. No platelets contamination (CD41⁺) within the RBC population was observed.

Rheological parameters of the total RBC population showed different tendencies according to the presence of mitochondria. Elmax was lower in the two groups with the highest percentages of mature RBC containing mitochondria, with a further reduction in the third (high) group (Figure 5A). The PoS was greater in patients with a high percentage of mature RBC with mitochondria compared to the two other groups (Figure 5B).

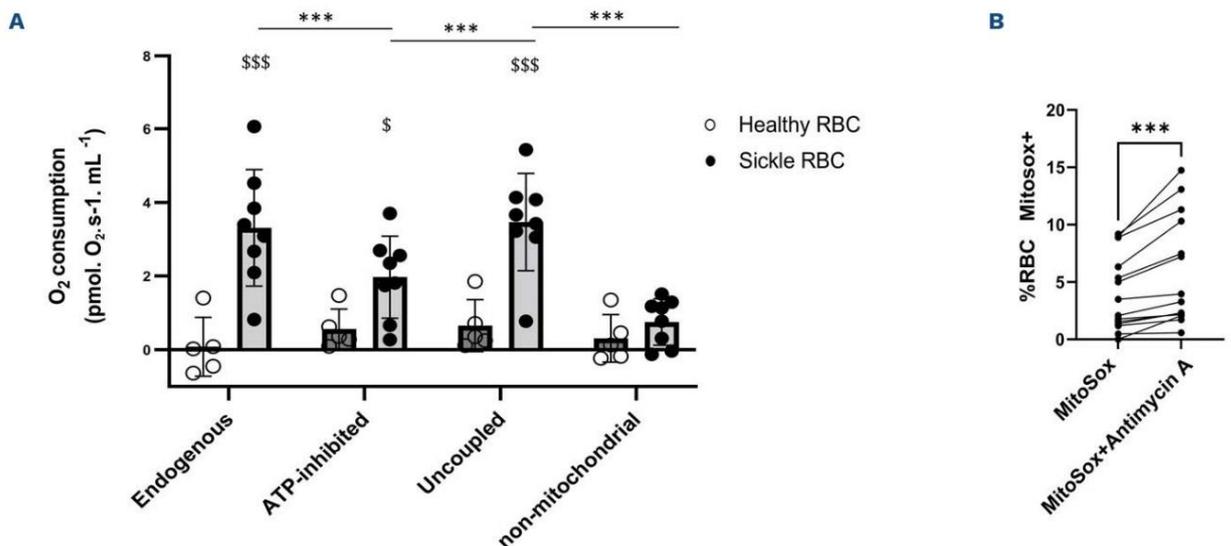


Figure 2. Mitochondrial respiration in mature red blood cells from sickle cell disease patients. (A) Comparison of mitochondrial respiration between 5 healthy and 8 mature sickle red blood cells (RBC). (B) Effect of Antimycin A on superoxide anion generated by mitochondria in mature sickle RBC (n=13). Endogenous: basal mitochondrial O_2 consumption; ATP-inhibited: mitochondrial O_2 consumption after the use of ATP-synthase inhibitor (Oligomycin); uncoupled: maximal uncoupled O_2 consumption obtained with the mitochondrial uncoupler (carbonyl cyanide-p-trifluoro-methoxyphenyl-hydrazone [FCCP]); non-mitochondrial: O_2 consumption after adding the Antimycin A. Difference between healthy and mature sickle RBC: * $P<0.05$; *** $P<0.001$. The addition of mitochondrial inhibitors or uncoupler had no effects on healthy RBC while changes were observed on mature sickle RBC: **** $P<0.001$. %RBC MitoSox⁺: percentages of RBC accumulating superoxide anion. Specify: \$, \$\$ and \$\$\$!

Hematocrit was lower and hemolytic markers were greater in patients with high percentages of mature red blood cells containing mitochondria

No difference in fetal hemoglobin (HbF) and mean corpuscular volume was observed between the three groups (Figures 6A, B). The mean corpuscular hemoglobin concentration was slightly but significantly increased in the group showing a higher percentage of mitochondrial retention compared to the group with a low percentage (Fig-

ure 6C). Percentages of reticulocytes and bilirubin levels were greater, and hematocrit was lower, in the group of patients with a high percentage of mature RBC with mitochondria (Figure 6D-F).

No association between mature sickle RBC mitochondria retention and previous history of clinical manifestations (i.e., vaso-occlusive crises and acute chest syndrome rates in the 3 preceding years, glomerulopathy, priapism, leg ulcers, osteonecrosis, stroke or pulmonary hypertension)

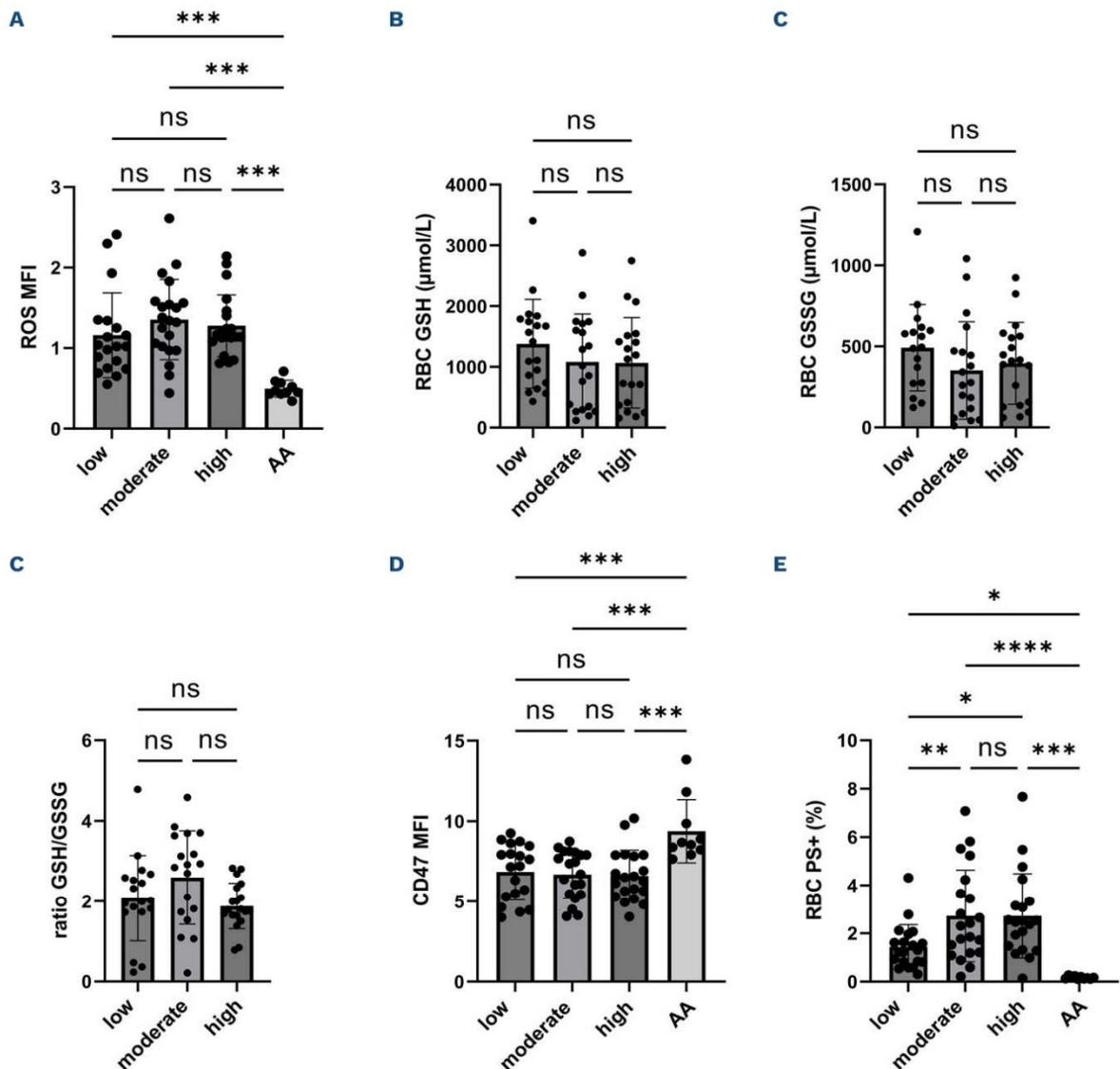


Figure 3. Comparisons of red blood cells oxidative stress and senescence markers between the three groups of sickle cell disease patients categorized according to the percentage of mature sickle red blood cell containing mitochondria (low group n=20, moderate group n=21, high group n=21) and healthy individuals (AA group n=10). (A-D) Red blood cell (RBC) oxidative stress markers, (E) CD47 expression and (F) percentages of RBC exposing phosphatidylserine (PS). ROS: reactive oxygen species; GSH: reduced glutathione; GSSG: oxidized glutathione; MFI: mean fluorescence intensity; ns: no significant difference. Significant difference: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

was observed. G6PD deficiency frequency did not differ between the three groups (15%, 14% and 10% in the low, moderate, and high group, respectively; $\chi=0.39$; $P= 0.82$).

Discussion

The present study investigated the functionality of mitochondria in mature RBC from SCA patients and the associations between RBC mitochondria retention, the propensity of RBC to sickle under deoxygenation, hemolytic markers, RBC senescence and RBC oxidative stress. Our findings support the fact that mitochondria in mature RBC from SCA patients are still functional and able

to produce ATP. In addition, we found that i) patients with a higher percentage of mitochondria retention in mature RBC were those exhibiting higher hemolytic rate, increased RBC senescence and greater propensity of RBC to sickle under deoxygenation, ii) oxidative stress and senescence markers were greater in mature sickle RBC containing mitochondria compared to those without.

Martino et al.⁵ reported that the abnormal retention of mitochondria in sickle RBC was the result of a lower expression of mitophagy inducers PINK1, NIX and of a higher expression of HSP90 chaperone. The interest in studying mitochondria retention in sickle RBC is based on the fact that these organelles may be a source of ROS

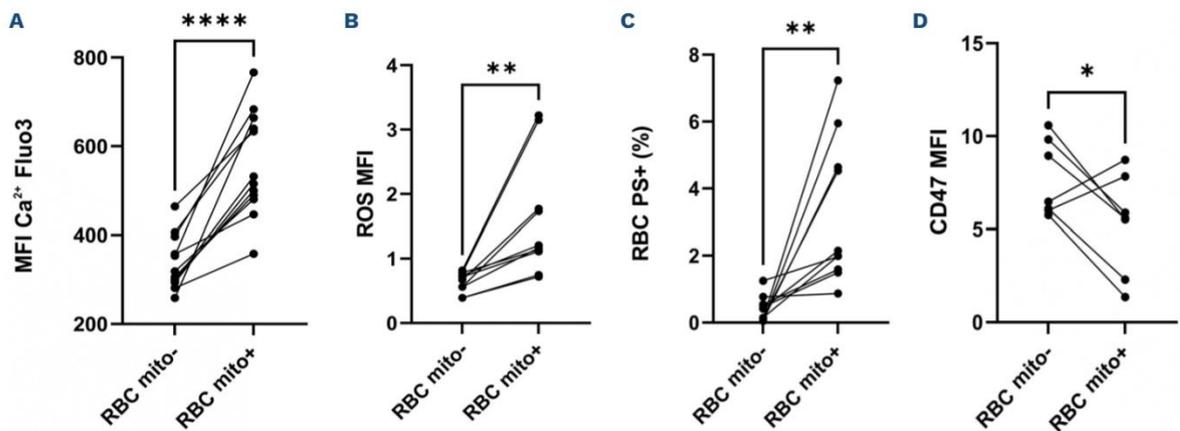


Figure 4. Comparisons of oxidative stress and senescence markers between mature sickle red blood cells containing mitochondria and mature sickle red blood cells without. (A) Ca²⁺ levels, (B) intracellular reactive oxygen species (ROS), (C) red blood cells (RBC) exposing phosphatidylserine (PS) and (D) CD47 expression. MFI: mean fluorescence intensity. mito: mitochondria. Significant difference: * $P<0.05$; ** $P<0.01$; *** $P<0.001$; **** $P<0.0001$.

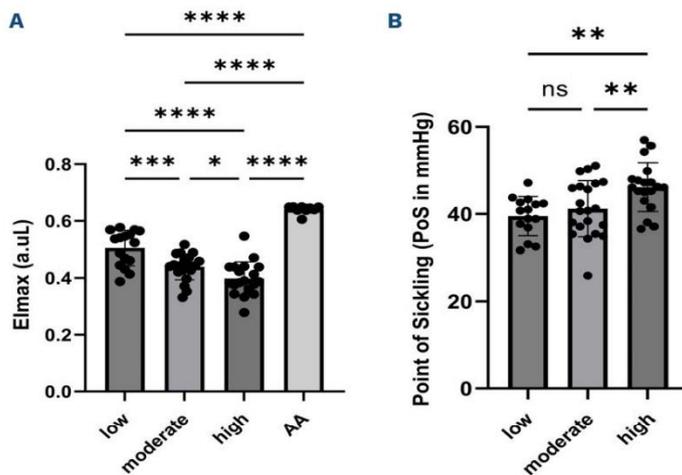


Figure 5. Comparisons of red blood cell rheological parameters between the three groups of sickle cell disease patients categorized according to the percentage of mature sickle red blood cells containing mitochondria (low group n=20, moderate group n=21, high group n=21) and healthy individuals (AA group n=10). (A) Elmax and (B) point of sickling (PoS). Elmax: red blood cell (RBC) deformability in normoxia; ns: no significant difference. Significant difference: * $P<0.05$; ** $P<0.01$; *** $P<0.001$; **** $P<0.0001$.

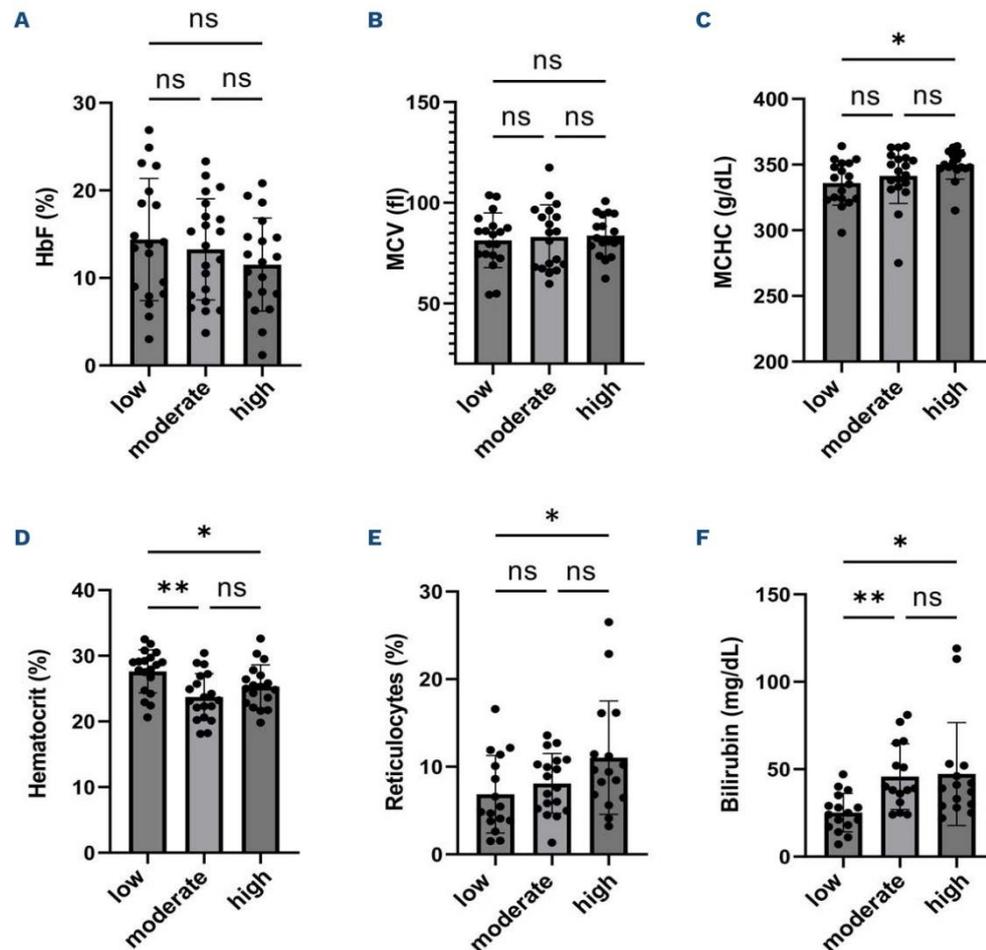


Figure 6. Influence of the presence of mitochondria in mature sickle red blood cells on hematological parameters. (A-D) Comparison of hematological parameters and (E-F) hemolytic markers between the 3 groups (low group n=20, moderate n=21, high n=21). HbF: percentage of fetal hemoglobin; MCV: mean corpuscular volume; MCHC: mean corpuscular hemoglobin concentration; ns: no significant difference. Significant difference: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

that could damage RBC and accelerate their death/removal from blood circulation. Our results did not show any difference in RBC oxidative stress markers between patients with high mitochondria retention in mature RBC and those with low retention. The lack of association between RBC oxidative stress level and the degree of mitochondria retention in mature RBC is in agreement with the study of Martino *et al.*⁵ However, the intracellular environment of sickle RBC is already characterized by a high level of oxidative stress caused by a high HbS auto-oxidation rate, increased NADPH oxidase activity and low antioxidant defense.¹⁷ In addition, the probe used to assess intracellular ROS in our study (i.e., DCFDA), although having several advantages, also has some limitations. DCFDA reacts with both nitric oxide and

ROS¹⁸ and DCF formation is increased in the presence of heme-containing molecules, like peroxidase, hematin or metal ions with redox action.^{19,20} Thus, even if the mitochondria in sickle RBC were functional, it is tempting to hypothesize that their contribution to the whole intracellular ROS production and RBC oxidative stress is too weak to be probed specifically by DCFDA. Nevertheless, the comparison of intracellular ROS levels between mature RBC containing mitochondria and mature RBC without, showed higher ROS content in the former RBC subpopulation. Indeed, the lack of intracellular ROS difference between the three groups could be due to the fact that the higher intracellular ROS content of the mature RBC containing mitochondria was diluted in the whole RBC population (i.e., mature RBC with and without

mitochondria retention). The use of a more specific probe for mitochondrial superoxide anion detection (i.e., MitoSox Red) indicated the capability of these RBC mitochondria to produce superoxide anions, especially after the incubation with Antimycin A, a blocker of mitochondrial complex III, which resulted in increased mitochondrial ROS accumulation. Indeed, we suspect that mitochondria in mature sickle RBC could contribute to the production of ROS and thus explain why inducing mitophagy in a sickle mice model resulted in a decrease of RBC ROS levels and increased RBC survival.⁹

The functionality of mitochondria in mature RBC from sickle cell patients was confirmed by the respiration assay experiments. Indeed, we observed a mitochondria-dependent oxygen consumption in sickle RBC and the use of oligomycin demonstrated that about 48.7% of endogenous oxygen consumption was related to the production of ATP. Image Stream experiments also showed that active mitochondria are present in sickle RBC, further supporting mitochondrial functionality in mature sickle RBC. Although we were not able to measure individually the deformability of mature sickle RBC with mitochondria and of mature sickle RBC without mitochondria, we observed that patients with a high percentage of mitochondria retention in mature RBC had a lower mean RBC deformability and a greater propensity of RBC to sickle under deoxygenation compared to patients with a lower percentage of mature RBC containing mitochondria. Indeed, it is possible that a higher rate of oxygen consumption by mitochondria contained in mature RBC from SCA patients could facilitate sickling by decreasing the amount of oxygen available for HbS. The subsequent greater rheological alterations would then increase RBC fragility,² which may explain the lower hematocrit and greater hemolytic rate found in the patients with high mitochondria retention in mature RBC, as previously reported.⁵ Moreover, the production of ROS by mitochondria could affect the RBC membrane and increase the risk of RBC membrane disruption. Alternatively, a high rate of hemolysis could induce abnormal erythropoiesis and altered RBC maturation process, which would lead in turn to a greater retention of mitochondria in mature RBC. Further studies are needed to investigate the mechanisms at the origin of the relationships between mitochondria retention in mature RBC and increased hemolysis in SCA patients.

Patients with a high percentage of mature RBC containing mitochondria exhibited higher percentages of RBC with externalized PS, which could indicate an alteration of membrane asymmetry following scramblase stimulation and accelerated RBC senescence. The comparisons of mature RBC subpopulations according to the presence or not of mitochondria showed a higher percentage of RBC with externalized PS in mature RBC with mito-

chondria retention and that mature RBC with mitochondria had greater level of intracellular Ca^{2+} and lower membrane CD47 compared to mature RBC with no mitochondria. Of note, the evaluation of the percentages of RBC exposing PS was assessed using Annexin V, a protein with a high affinity for PS but that could also enter cells with membrane alterations and bind PS on the inner leaflet. This could have led to an overestimation of external PS and the use of lactadherin should be considered in future studies.

Increased cation permeability, and more particularly of Ca^{2+} , has been reported in sickle RBC compared to normal RBC,²¹ notably in deoxygenated condition.²² Hanggi *et al.*²³ reported increased Ca^{2+} conductance through NMDA receptors in sickle RBC and Wang *et al.*²⁴ demonstrated increased Ca^{2+} accumulation in response to lysophosphatidic acid in sickle RBC compared to control RBC. Very recently, an important role of Piezo1 has been reported in SCA that may increase intracellular Ca^{2+} levels in sickle RBC.²¹ Intracellular accumulation of Ca^{2+} is known to stimulate scramblase, leading to a disruption of the membrane phospholipids asymmetry and PS externalization.²⁵ The presence of mitochondria could also participate in the elevation of intracellular Ca^{2+} levels, as mitochondria have a remarkable ability to take up and store massive amounts of Ca^{2+} ,²⁶ as well as in the release of it into the cytosol.²⁷ Although mitochondria would provide RBC with ATP, the low residual mitochondria in sickle RBC are unable to maintain ATP levels high enough to ensure Ca^{2+} ATP-dependent extrusion.

It is well known that reticulocytes still have the endoplasmic reticulum, which is a major source of Ca^{2+} .²⁸ Reticulocytes normally lose all their remaining organelles during maturation. The abnormal retention of mitochondria and the increased Ca^{2+} and PS exposure in mature RBC could point to a defect in the final maturation of reticulocytes.²⁹ Further studies are needed to analyze the content in endoplasmic reticulum proteins²⁹ in mature RBC in SCA. Furthermore, Ca^{2+} may cause the activation of the Gardos channel pathway, which mediates rapid K^+ and Cl^- efflux and water loss.³⁰ The resulting cellular dehydration facilitates HbS polymerization and RBC sickling, which could explain why SCA patients with the greatest percentage of mature RBC containing mitochondria were also those with a lower deformability, a greater MCHC and a higher point of sickling, i.e., with a greater propensity of RBC to sickle under deoxygenation. In conclusion, this study showed the presence of functional mitochondria in mature sickle RBC, which could favor RBC sickling and accelerate RBC senescence, leading to increased cellular fragility and hemolysis.

Disclosures

No conflicts of interest to disclose.

Contributions

SE, EN, AS, RVB, CB, DR and PC designed the research. SE, EN, AS, CB, RC, MM, MR, CB, CR, PJ and DR performed the biological analyses. AG and SP included patients. SE, EN, AS and PC performed the statistical analyses. SE, EN, AS and PC wrote the first draft of the paper. CB, RC, MM, MR, FH, AC, CR, RVB, CB, YD, PJ, AG, SP, MR and DR read and approved the final version of the manuscript.

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Data-sharing statement

Data are available upon reasonable request to the corresponding author.

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7.3 Study n°3

Mitochondria retention in mature RBCs from

Hemoglobin SC patients

Esperti S, Nader E, Boisson C, Carin R, Horrand F, Piedrahita D, Renoux C, Joly P, Gauthier A, Poutrel S, Connes P. Mitochondria retention in mature RBCs from haemoglobin SC patients. *Br J Haematol*. 2023 Jun 12. Doi: 10.1111/bjh.18935. Epub ahead of print. PMID: 37305963.

HbSC disease is usually characterized by milder anemia and clinical complication compared to SCA. Furthermore, if an abnormal mitochondria retention has been reported in mature RBCs from SCA patients as the result of a deficient mitophagy pathway, no data about the presence of mitochondria within RBCs from HbSC patients have been published so far.

In this study, we analyzed for the first time the presence of mitochondria-positive RBCs in patients with HbSC disease and we investigated the associations between mitochondria retention into mature RBCs and RBC deformability, senescence markers, oxidative stress, hemolytic markers, and clinical complications.

Sixteen HbSC patients and sixteen healthy donors participated to this study. HbSC patients were at steady state, without any recent blood transfusion or clinical complications in the last 3 months. A group of 16 SCA patients has been included to compare the parameters investigated. Fresh blood samples were analyzed by flow cytometry and the presence of residual mitochondria has been assessed using the Mitotracker deep red probe and an anti-reticulocytes antibody (anti CD71) to discriminate mitochondria from mature RBCs and reticulocytes. We also tested for the Phosphatidylserine exposure using the Annexin V and for intracellular reactive oxygen species (ROS) using 2',7'-dichlorofluorescein diacetate (DCFDA). Rheological properties of RBCs were assessed using the LORRCA protocol to evaluate RBCs deformability (Elongation Index, EI) in isotonic medium in a range of shear stress from

0.3 to 30 Pa, and Lactate dehydrogenase plasma concentration was analyzed using standard biochemical protocols. The results showed very low levels of RBCs retaining mitochondria in healthy RBCs compared to HbSC RBCs. However, when compared to SCA patients, HbSC patients showed lower levels of RBCs retaining mitochondria. The presence of mitochondria was associated to decreased RBC deformability in HbSC patients. Different levels of RBCs exposing PS were observed between the three groups, with higher levels of exposed PS in SCA RBCs compared to HbSC and healthy RBC. The presence of mitochondria into RBCs correlated with the percentage of RBCs positive for PS. No correlation between the presence of mitochondria in HbSC RBCs and oxidative stress, hemolysis or clinical manifestations were observed.

The results suggest that, as observed in SCA patients, the mitochondria retention within HbSC RBCs is associated a decrease in RBC deformability and increased RBC senescence. However, we have no information about the functionality of the residual mitochondria in HbSC RBCs. It is tempting to hypothesize that they could be still functional, as the Mitotracker staining depends on the mitochondrial membrane potential, but further investigations are needed to address this point.

LETTER TO THE EDITOR

Mitochondria retention in mature RBCs from haemoglobin SC patients

To the Editor,

Sickle cell disease (SCD) defines a group of hemoglobinopathies affecting the β -globin gene. Sickle cell SC disease (HbSC) is a SCD syndrome characterized by the presence of both haemoglobin S (HbS) and haemoglobin C (HbC) in red blood cells (RBCs).¹ HbS is caused by a single point mutation in the β -globin gene, responsible for the substitution of valine for glutamic acid. HbS may polymerize upon deoxygenation.² HbC is another abnormal haemoglobin and is caused by a single point mutation in the same position of the β -globin gene as for HbS, but with a lysine replacing glutamic acid.³ HbC has the tendency to crystallize in both oxygenated and deoxygenated conditions and promotes RBC dehydration.^{4,5} HbSC patients are usually characterized by milder anaemia than the homozygous form of SCD (i.e. HbSS), low RBC deformability and very high blood viscosity.⁶ Recently, abnormal mitochondria retention has been reported in mature RBCs from HbSS patients, which correlated with accelerated RBC senescence and hemolysis.⁷ In another recent study, Esperti et al.⁸ reported an association between mitochondria retention in mature RBCs, RBC senescence and RBC sickling in HbSS individuals. However, no data about the presence of mitochondria in mature RBCs from HbSC patients have been published so far. In this study, we tested for the presence of mitochondria-positive RBCs in patients with HbSC disease and investigated the association between mitochondria retention into mature RBCs and RBC deformability, senescence, oxidative stress, haemolytic markers and clinical manifestations.

Fresh blood samples from 16 healthy donors and 16 HbSC individuals (adults and children at steady state, non-transfused, without clinical complications in the last 3 months before this study) were collected in EDTA tubes and analysed by Flow cytometry less than 5 h after sampling. A group of 16 HbSS patients was also included for the comparisons of selected parameters. All patients were regularly followed at the Hospitals of Lyon (France). The study was conducted in accordance with the guidelines set by the Declaration of Helsinki and approved by the Regional Ethics Committees (L14-127, Université de Lyon). Blood samples were washed twice in PBS 1X (1:1) at 1000g for 5 min at room temperature (RT). RBCs pellets were resuspended at 0.2% Ht in PBS 1X (1:1) or in the appropriate staining buffer according to manufacturer instructions. The percentage of mature

RBCs retaining mitochondria was assessed by flow cytometry performing a double staining using MitoTracker^(R) Deep Red probe (MTKdr; Sigma-Aldrich; 5 nM final concentration) and an anti-CD71 antibody (1:50 dilution, Miltenyi 130-115-070), to discriminate the percentages of reticulocytes and mature RBCs retaining mitochondria. The percentage of positive events was recorded to quantify mitochondria⁺ and CD71⁺ RBCs. Phosphatidylserine (PS) exposure on the outer membrane of RBCs was assessed incubating RBCs with Annexin V-PE (1:11 dilution, Miltenyi 130-118-363) and intracellular reactive oxygen species (ROS) were determined using 2',7'-dichlorofluorescein diacetate (DCFDA, 20 μ M final concentration, Sigma-Aldrich). After incubation (20 min in the dark at RT), the samples were washed, resuspended in their respective staining buffer and analysed by flow cytometry (MACSQuant 16, Miltenyi). Ektacytometry was performed with the Laser-assisted optical rotational red cell analyser (LORRCA, Mechatronics) to evaluate RBC deformability (Elongation Index, EI) in isotonic medium (Polyvinylpyrrolidone, PVP) over a shear stress gradient (0.3–30 Pa). Lactate dehydrogenase levels were determined by the standard biochemical method.

The results showed no or very low levels of mitochondria-positive mature RBCs in healthy donors, compared to RBCs from HbSC patients (Figure 1A). The level of mitochondria-positive mature RBCs was lower in HbSC compared to HbSS patients (Figure 1A). RBC deformability was lower in both HbSC (0.41 ± 0.07) and HbSS (0.36 ± 0.06) patients compared to healthy individuals (0.64 ± 0.005 ; $p < 0.0001$). The percentages of mature RBCs with mitochondria negatively correlated with RBC deformability in HbSC patients (Figure 1B). The level of RBCs exposing PS was different between the three groups (HbSS = 3.03 ± 1.86 ; HbSC = 1.4 ± 1.1 %, healthy individuals = 0.2 ± 0.08 %; $p < 0.0001$).

Several associations were tested in HbSC patients. A positive correlation between the percentage of mature RBCs retaining mitochondria and the PS exposure was observed (Figure 1C). No significant correlation was observed between the percentage of mature RBCs retaining mitochondria and intracellular ROS levels ($r = -0.17$; $p > 0.05$), LDH ($r = -0.01$; $p > 0.05$), percentages of reticulocytes ($r = -0.03$; $p > 0.05$) or haematocrit ($r = 0.20$; $p < 0.05$). No association between mature sickle RBC mitochondria retention and previous history of clinical manifestations (i.e. vaso-occlusive crises

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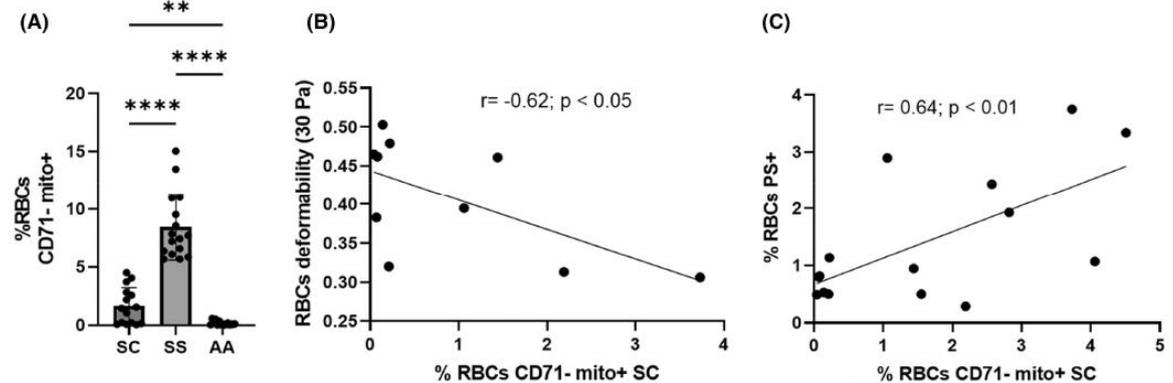


FIGURE 1 Comparison of mitochondria retention into mature RBCs (%RBCs mito+) between HbSC patients, HbSS patients and healthy donors (AA; A). Correlations between the percentages of mature RBCs containing mitochondria and RBC deformability (B, $n = 10$) or percentages of RBCs with externalized PS (%RBCs PS+; C, $N = 14$) in HbSC patients. Significant difference: ** $p < 0.01$; *** $p < 0.0001$.

and acute chest syndrome rates in the three preceding years, glomerulopathy, priapism, leg ulcers, osteonecrosis, stroke or pulmonary hypertension) was observed.

During the terminal erythroid differentiation the removal of mitochondria is ensured by a specific autophagy pathway, called mitophagy. The lack of mitochondria-positive RBCs in healthy controls is in agreement with previous studies.^{7,8} However, recent studies reported mitochondria retention in mature RBCs in HbSS patients, probably as a consequence of deficient mitophagy.^{7,9,10} Mitochondria are a source of reactive oxygen species (ROS), which could contribute to increase the intracellular oxidative environment of RBCs, which in SCD is already increased.¹¹⁻¹³ In a sickle mice model, the pharmacological inhibition of mitochondria retention has been correlated to decreased intracellular ROS levels and improved RBCs survival.¹⁴

Our results show for the first time abnormal mitochondria retention in RBCs from HbSC patients, although the extent of mitochondrial retention is lower compared to HbSS individuals. The reasons for such retention are unknown and whether it is due, as for HbSS patients, to deficient mitophagy remains unknown. Although we did not find any association between mitochondria retention into mature RBCs and hemolytic markers or the level of anaemia, our findings suggest that the retention of mitochondria into mature RBCs could promote senescence, as demonstrated by the associations between the percentages of mature RBCs with mitochondria and RBC deformability and PS externalization. The accumulation of intracellular ROS, as well as Ca^{2+} released by mitochondria,¹⁵ into the cytosol of RBCs could accelerate senescence. These findings reinforce the role that abnormal mitochondrial retention could play in modulating RBC properties and sickling in SCD, as recently shown in HbSS patients.⁸

Further investigations about the functionality of these residual mitochondria in HbSC RBCs, as well as the clinical relevance of such findings, are needed.

AUTHOR CONTRIBUTIONS

Sofia Esperti, Elie Nader, Camille Boisson and Philippe Connes designed the research. Sofia Esperti, Elie Nader, Camille Boisson, Romain Carin, Céline Renoux and Philippe Joly performed the biological analyses. Alexandra Gauthier and Solène Poutrel included patients. Sofia Esperti, Elie Nader and Philippe Connes performed the statistical analyses. Sofia Esperti, Elie Nader and Philippe Connes wrote the first version of the paper. Camille Boisson, Romain Carin, Françoise Horrand, Céline Renoux, Philippe Joly, Alexandra Gauthier and Solène Poutrel read and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

No conflict of interest to declare.

ETHICS APPROVAL STATEMENT

The study was conducted in accordance with the guidelines set by the Declaration of Helsinki and approved by the Regional Ethics Committees (L14-127, Université de Lyon).

DATA AVAILABILITY STATEMENT

Data are available upon reasonable request to the corresponding author.

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7.4 Study n°4

Piezo1 activation augments sickling propensity and the adhesive properties of sickle red blood cells in a calcium-dependent manner.

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Cation permeability plays a crucial role in the modulation of RBC dehydration and sickling in SCA. This increased permeability is named P_{Sickle} but the protein involved has not been clearly identified. In this study, the mechanosensitive cation channel Piezo1 has been proposed to be a candidate for P_{Sickle} , which promotes the influx of Ca^{2+} into the cell, causing Gardos channel activation and cell dehydration.

Here we investigated the role of Piezo1 in Gardos channel activity, RBC sickling, senescence, and adhesion. Patients from Hospices Civils de Lyon and Institut d'Hématologie et d'Oncologie Pédiatrique de Lyon and from The Hematology Center of the University of Campinas participated to this study.

We analyzed the impact of a Piezo1 pharmacological activator (Yoda1) or an inhibitor (GsMTx4) on RBC sickling, RBC rheological, RBC adhesion to laminin and senescence markers. The effect of Yoda1 on the changes in the membrane potential was also studied. The use of TRAM34 molecule (Gardos channel inhibitor) allowed us to make a link between Piezo1 modulation, Gardos channel activation and changes in RBC rheology. We also compared RBCs parameters between SCA patients with a gain of function mutation in Piezo1 and those without.

The results showed that increasing the activity of Piezo1 in sickle RBCs lead to pronounced membrane hyperpolarization. The magnitude of the response was further higher during deoxygenated versus oxygenated conditions. The accumulation of Ca^{2+} following Piezo1 activation led to Gardos channel activation, which resulted in a decrease in RBC deformability and an increased propensity of RBCs to sickle at

higher partial oxygen pressure, as well as an increased RBC adhesiveness in a Ca^{2+} and BCAM-dependent manner. The use of Yoda1 also increased PS exposure on the outer membrane of RBCs and decreased the expression of the antiphagocytic antigen CD47. Opposite results concerning the rheology of sickle RBCs were observed using the Piezo1 inhibitor GsMTx4. A comparison of SCA patients carrying the gain of function mutation (E756del) to those without demonstrated a difference in the propensity of RBCs to sickle upon deoxygenation.

Our results suggest a key role of Piezo1 in the alteration of sickle RBCs properties, such as decreased RBCs deformability and increased propensity to sickle in deoxygenated conditions. Furthermore, Piezo1 stimulation was observed to modulate the sickle RBCs adhesive interactions to Laminin, an extracellular matrix molecule, and to promote sickle RBCs senescence.

Piezo1 activation augments sickling propensity and the adhesive properties of sickle red blood cells in a calcium-dependent manner

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Summary

Haemoglobin S polymerization in the red blood cells (RBCs) of individuals with sickle cell anaemia (SCA) can cause RBC sickling and cellular alterations. Piezo1 is a mechanosensitive protein that modulates intracellular calcium (Ca^{2+}) influx, and its activation has been associated with increased RBC surface membrane phosphatidylserine (PS) exposure. Hypothesizing that Piezo1 activation, and ensuing Gárdos channel activity, alter sickle RBC properties, RBCs from patients with SCA were incubated with the Piezo1 agonist, Yoda1 (0.1–10 μ M). Oxygen-gradient ektacytometry and membrane potential measurement showed that Piezo1 activation significantly decreased sickle RBC deformability, augmented sickling propensity, and triggered pronounced membrane hyperpolarization, in association with Gárdos channel activation and Ca^{2+} influx. Yoda1 induced Ca^{2+} -dependent adhesion of sickle RBCs to laminin, in microfluidic assays, mediated by increased BCAM binding affinity. Furthermore, RBCs from SCA patients that were homo-/heterozygous for the rs59446030 gain-of-function Piezo1 variant demonstrated enhanced sickling under deoxygenation and increased PS exposure. Thus, Piezo1 stimulation decreases sickle RBC deformability, and increases the propensities of these cells to sickle upon deoxygenation and adhere to laminin. Results support a role of Piezo1 in some of the RBC properties that contribute to SCA vaso-occlusion, indicating that Piezo1 may represent a potential therapeutic target molecule for this disease.

KEYWORDS

adhesion molecule, calcium influx, erythrocyte, rheology, vaso-occlusion

INTRODUCTION

Sickle cell disease (SCD) is a group of inherited disorders, caused by mutations in the β -globin gene, where sickle cell

anaemia (SCA, HbSS) constitutes the homozygous form of SCD. SCD is characterized by the production of an abnormal haemoglobin (Hb), called HbS, which may polymerize when deoxygenated, causing mechanical distortion (i.e. sickling) of

Elie Nader and Nicola Conran share the same position.

Stephane Egee and Philippe Connes share the same position.

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red blood cells (RBCs).¹ Sickled RBCs are poorly deformable and very fragile, whereby the pathophysiological changes incurred from these alterations result in several acute and chronic complications, including enhanced intravascular and extravascular haemolysis, anaemia, frequent painful vaso-occlusive episodes, auto-splenectomy, acute chest syndrome, priapism, pulmonary hypertension, leg ulcers, nephropathy, retinopathy, hepatopathy, osteonecrosis and stroke.^{2,3}

Activation of the Gárdos pathway upon deoxygenation has been shown to play a key role in the dehydration of RBCs from SCA patients, as well from patients with other SCD genotypes (HbSC for instance), further increasing the propensity of HbS to polymerize and RBCs to sickle.⁴ Increased RBC Ca^{2+} , due to permeability caused by deoxygenation and sickling, causes the loss of K^+ through the Gárdos channel. This increased Ca^{2+} influx, also known as P_{sickle} , is considered to be the manifestation of a non-selective cation conductance for monovalent and divalent cations such as Na^+ , K^+ , Rb^+ , Ca^{2+} and Mg^{2+} .⁵⁻⁷ At this time, the protein (or proteins) that mediates P_{sickle} has not been identified, but the inhibitory effect of *Grammastola spatulata* mechanotoxin-4 (GsMTx4) suggests that P_{sickle} could be a mechanosensitive protein,^{8,9} and it has been proposed that Piezo1 could be a good candidate for P_{sickle} .^{8,10,11} Piezo1 (also known as FAM38A) is a protein with mechanosensitive properties that is involved in the cellular influx of calcium (Ca^{2+}). Genetic mutations, considered as gain of function, affect Piezo1 deactivation and thus increase its opening time, leading to an uncontrolled influx of Ca^{2+} that will secondarily activate the Gárdos channel. Such mutations have been associated with hereditary xerocytosis, explaining the characteristic dehydrated state of RBCs in this disease.^{10,12} Indeed, one could speculate on a role for Piezo1 in RBC sickling in SCA; slight mechanical deformation, compatible with that encountered by RBCs in the circulation, can produce robust cellular Ca^{2+} entry that is strictly dependent on Piezo1 expression.^{11,13} SCA pathophysiology is characterized by the occurrence of continuous vaso-occlusive processes in the microcirculation that result from a complex interplay between altered blood rheology, inflammatory processes and the adhesion of RBCs, leukocytes and platelets to each other and to the endothelium.¹⁴ While sickle RBCs display an augmented expression of multiple cell surface adhesion molecules,¹⁵ modifications in the membrane topography induced by the accumulation of Ca^{2+} in sickle RBCs could be accompanied by increased adhesiveness,¹⁶ even at a concentration below the threshold required for cell dehydration. A recent study found that Piezo1 activation on sickle RBCs could induce Ca^{2+} entry into cells and increase phosphatidylserine (PS) exposure.¹⁷ While calcium accumulation has been reported to promote intercellular adhesion interactions between red blood cells under conditions of strength consistent with those encountered within the circulation,¹⁸ the impact of Ca^{2+} accumulation in RBCs on adhesion to endothelial cells/extracellular matrix ligands is still unknown. We hypothesize that Piezo1 could, thus, be involved in the modulation of sickle RBC properties such as cell sickling and adhesive properties.

The aim of this project was to characterize the role of Piezo1 and ensuing Gárdos channel activity in RBC sickling and adhesion in SCA. We used pharmacological tools to inhibit or activate Piezo1 in sickle RBCs, in the presence of conditions mimicking shear stress and/or deoxygenation, and investigated the consequences of channel activity modulation on the rheological properties, sickling, senescence markers, membrane potential and adhesive properties of sickle RBCs. We also screened frequent polymorphisms of the *PIEZO1* gene in SCA patients and tested for their associations with blood rheological parameters.

METHODS

SCA patients

Patients (HbSS genotype) were recruited, with informed consent, from Hospices Civils de Lyon and Institut d'Hématologie et d'Oncologie Pédiatrique de Lyon, France, and from the Hematology Center, University of Campinas, Brazil. Healthy controls participated in the study after giving written informed consent. All subjects were in clinical steady-state at the time of the study; that is, without experiencing any vaso-occlusive crisis or other acute medical complication within the last 2 months, and without any blood transfusions for at least 3 months before inclusion. The study was conducted in accordance with the guidelines set by the Declaration of Helsinki and approved by the Regional Ethics Committees (L14-127, Université de Lyon; 4.649.745, University of Campinas).

Preparation and treatment of red blood cells

RBCs from SCA patients (SS RBCs) were separated from peripheral blood samples (collected in citrate) by centrifugation (800 g, 10 min, 20°C). RBCs were washed in PBS 1×, and RBC pellets were resuspended in PBS buffer containing 2.5 mM Ca^{2+} and incubated (30 min, 37°C) with Yoda1 (Sigma Aldrich or Saint-Quentin-Fallavier; 0.1–10 μM, depending on the assay), a Piezo1 agonist, or GsMTx4 (Sigma Aldrich; 10 μM), a Piezo1 antagonist, or vehicle (dimethyl sulfoxide, DMSO; equivalent dilution) before assays. In some assays, cells were preincubated with BAPTA AM (Tocris; a cell-permeable calcium chelator; 10 μM) for 30 min, or adhesion molecule neutralizing antibodies (25 μg/mL anti-BCAM [AF148]; 10 μg/mL anti-VLA4 [clone 2B4], R&D Systems). All assays were performed on cells in the continued presence of agonists/antagonists/inhibitors/antibodies following pre-incubation.

Flow cytometry

Following the 30 min incubations with Yoda1 or GsMTx4, PS exposure on the outer membrane leaflet of the RBCs was

evaluated using Annexin V-FITC binding. RBC suspensions (in 0.4% Hct in PBS containing 2.5 mM Ca^{2+}) were protected from light and incubated for 30 min at room temperature (RT) with Annexin V-FITC (1:200 dilution, Beckman Coulter). As a PS-negative control, cells were incubated in the presence of 5 mM EDTA. Immediately after incubation, samples were diluted and analysed by FACS (BD Accuri C6 or BD Calibur). PS exposure was measured in the FITC channel (with an excitation wavelength of 488 nm and an emission wavelength of 530 nm), according to the manufacturer's instructions. The RBC surface expressions of CD47, CD49 and BCAM were also evaluated by flow cytometry (incubations, as above) using the anti-CD47-PE (1:33 dilution, Miltenyi Biotec), or anti-CD49-PE (clone ALC1/1; Abcam) and/or anti-CD239(BCAM)-APC (clone REA276, 1:11 dilution Miltenyi Biotec) antibodies. For each sample, 50 000 events were acquired and gated for the appropriate forward scatter (FSC)/ side scatter (SSC).

Intracellular calcium (Ca^{2+}) measurement

Intracellular RBC Ca^{2+} level was determined using Fluo3/AM. RBC suspensions (0.4% Hct) were incubated for 30 min at RT in the dark with 10 μM of Fluo3/AM (Biotium) probe. The samples were then analysed using flow cytometry, according to the manufacturer's instructions (BD Accuri C6). The median fluorescence intensity (MFI) of 50 000 gated events was recorded to quantify Ca^{2+} levels.

Microfluidic adhesion assays

RBC adhesion and rolling on laminin-coated microchannels (400 μm -wide microchannels, Cellix Ltd) were observed using a microfluidic platform (Cellix Ltd). Biochip channels were coated with 4 $\mu\text{g}/\text{mL}$ human laminin (LN, from human placenta, Sigma Aldrich) for 2 h at RT. The LN ligand concentration was chosen based on standardization assays, employing ligand concentrations of 2–10 $\mu\text{g}/\text{mL}$ (data not shown). Non-specific binding sites on channels were then blocked with 1% (w/v) BSA (30 min, RT) and chips were stored in a humidified chamber for use in assays within 1 h. After treatments, cell suspensions were loaded sequentially, onto microchannel entry wells, and a shear stress of 0.25 dyn/cm^2 applied for >3 min (ExiGo Pump Pulse-Free Microfluidic Syringe Pump). Images for each channel, in 3 fields, were acquired (1 per second) for video manufacture using a Zeiss microscope ($\times 40$ lens) and Exi Blue Cooled camera (Qimaging). Numbers of cells rolling along the chip (defined as a significant decrease in cell velocity through the channel, to 50% or less) and adhered to the chip were evaluated in 3 fields at the time of 180 s (3 min) post cell loading. The optimal perfusion time and shear stress for the assay were standardized previously and adapted to the current study conditions.¹⁹

Ektacytometry

After the 30 min incubations with the drugs modulating Piezo1 activity, RBC deformability was measured under an oxygen gradient by oxygenscan ektacytometry, as previously described.^{20–22} A volume of 50 μL of blood, standardized to a fixed total RBC count of 200×10^6 , was mixed with 5 mL of Oxy-Iso polyvinylpyrrolidone (PVP) suspension with a mean viscosity of 28–30 cP (Mechatronics, The Netherlands) and osmolality of 282–286 mOsm/kg. The suspension was sheared at 30 Pa and 37°C while the oxygen partial pressure (pO_2) was gradually decreased from 160 to 20 mm Hg (deoxygenation) and then returned to normoxic values. The diffraction pattern was analysed by computer to determine the maximum RBC deformability in normoxia (Elmax), the minimum RBC deformability reached during deoxygenation (Elmin), and the oxygen partial pressure at which RBC start to sickle (i.e. the Point of Sickling, PoS). PoS is defined as a decrease of at least 5% of Elmax during deoxygenation.^{20–22}

Membrane potential measurements

The CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) method^{23–25} or MBE method (as renamed recently)²⁶ was used for the monitoring of membrane potential evolution. Briefly, when RBCs are suspended in nominally buffer-free solution in the presence of the CCCP protonophore (10 μM), changes in extracellular pH reflect membrane potential changes since protons are kept at equilibrium across the membrane. The membrane potential (V_M) can, thus, be estimated from:

$$V_M = 61.51 \text{ mV} \cdot (\text{pH}_i - \text{pH}_o).$$

Due to the high red cell buffer capacity, the intracellular pH (pH_i) remains constant (at about 7.2) throughout an experiment and can be estimated as the pH of the solution after lysis with Triton™ X-100 at the end of the experiment. Regarding the experimental procedure, 2900 μL of experimental solution containing 20 μM of CCCP were heated to 37°C under constant magnetic stirring. For each experiment, 100 μL of packed RBCs (99% hct) were added, to reach a final cyto-crit of 3.3%. All inorganic compounds were added as stock solution 1000 \times in DMSO. The final concentration of DMSO never exceeded 0.3%, a concentration that has no effect on either fluxes or membrane potential. Extracellular pH was measured using a G200 pH electrode (Radiometer) coupled to a Red Rod 200 reference electrode (Radiometer) and a PHM210 pHmeter (Radiometer). Sampling and acquisition were done with an electrode amplifier (EA-BTA, Vernier) at a rate of 1 measurement per second connected to an AD LABQUEST Mini interface (Vernier) with a resolution of 0.01 pH unit. The data were visualized and analysed with the Logger Lite software (Vernier). At the end of each experiment, Triton X-100 detergent (1% in 3 M NaCl) was added,

causing total cell lysis and a resulting solution that attains the intracellular pH.

Next-generation sequencing

Genomic DNAs from 50 SCA patients were tested by next-generation sequencing (NGS) sequencing using a custom design based on a SeqCap EZ Solution-Based Enrichment strategy (Roche NimbleGen). Targeted sequencing capture probes were designed by Roche NimbleGen, in order to sequence the coding sequences (exons ± 30 pb) of the *PIEZO1* gene (NM_001142864). For each sample, 100 ng of high quality genomic DNA was used. Library preparation was performed with the Kapa HyperPlus Kit for Illumina platforms (Kapa Biosystems). The manufacturer's DNA sample preparation protocol for Roche NimbleGen SeqCap EZ Library (Roche, SeqCap-EZ_UGuide_v5p0) was followed, using dual index adapters. Pre-enrichment pools were performed by equimolarly pooling 24 samples. The patients were further sequenced on a MiSeq[®] sequencer using the MiSeq reagent kit v2 500 cycles (Illumina). FASTQ files (8 per patient) were uploaded and bioinformatic analyses were further performed using an academic pipeline.

For each sample, read pairs were first trimmed using trimomatic v 0.33.²⁷ Then, reads were aligned against the hg19 version of the human genome using BWA-MEM v 0.7.12,²⁸ producing a BAM file for each sample, which was indexed and sorted with samtools v 1.3.1.²⁹ This last then underwent several treatments: (i) duplicate marking was performed by PicardTools MarkDuplicates v 1.138 (Picard Toolkit, 2018); (ii) indel realignment and (iii) nucleotide recalibration were done using respectively GATK IndelRealigner and GATK BaseRecalibrator.³⁰ FASTQ and BAM metrics were collected using respectively FastQC³¹ and PicardTools (Picard Toolkit software, 2018). The variant calling was performed using GATK HaplotypeCaller,³⁰ producing a genomic VCF for each sample. The genotyping was performed using GATK GenotypeGVCFs,¹⁵ merging all the samples in a unique VCF. Variants normalization and annotation were respectively handled by GATK LeftAlignAndTrimVariants¹¹ and the snpEff/SnpSift toolbox.³² VCF metrics were collected using snpEff/SnpSift.³² DeCovA, an in-house script, was used for copy number variant detection.³³ Identitovigilance process was done using SNPplex technology. Only frequent (> 10% in the SCA patients) single nucleotide variations (SNV) were selected. The PoS and RBC PS exposure were also measured in these patients.

Data analysis

Data are depicted as means \pm SEM; normal distribution of data was confirmed, or not, and groups were statistically compared using paired *t*-tests or one way RM ANOVA completed by Friedman multiple comparison test, Sidak's multiple comparison test or Dunn's multiple comparison test, as

appropriate. Pearson correlation was performed to test the association between PoS and changes in membrane potential following Piezo1 activation. A *t*-test was used to compare healthy individuals with SCA patients. A $p < 0.05$ was considered as significant. GraphPad Prism 7 software was used for statistical analyses.

RESULTS

Piezo1 activity modulates RBC deformability and sickling in SCA

Suspensions of RBCs from SCA patients (SS RBCs) were subjected to pharmacological modulation of Piezo1 channel activity with Yoda1 (Piezo1 agonist) or GsMTx4 (Piezo1 antagonist) as described in Section 2, and RBC deformability and sickling propensity were investigated using oxygen gradient ektacytometry (Figure 1). Figure S1 shows a typical example of the RBC deformability response with both molecules. Piezo1 activation (Yoda1, 10 μ M) significantly decreased the deformability of SS RBCs under normoxia and the SS RBCs started to sickle at higher oxygen partial pressures (Figure 1A,C). Similarly, the inhibition of Piezo1 activation with GsMTx4 (10 μ M) increased SS RBC deformability in normoxia and lowered the oxygen partial pressure (i.e. PoS) at which the cells started to sickle (Figure 1B,D). Piezo1 activation by Yoda1 incubation (0.1–5 μ M; 30 min 37°C) also induced significant visible SS RBC sickling under conditions of flow (0.25 dyn/cm²), as evaluated microscopically on a microfluidic platform (Figure 1E; cell sickling status was observed for 3 min in 400- μ m-wide channels). The deformability (EImax) of RBCs from healthy individuals without SCA (AA RBC, $n=8$) was higher compared to SCA patients ($p < 0.001$), decreased from 0.63 ± 0.1 to 0.56 ± 0.03 ($p < 0.001$) with Yoda1 (10 μ M) and did not change with GsMTx4 (0.62 ± 0.02).

Piezo1 modulates sickle RBC adhesion to laminin in a BCAM-dependent manner

Having confirmed that Piezo1 activation reduces SS RBC deformability and augments sickling, we looked at whether Piezo1 activation could modulate SS RBC adhesive interactions with an extracellular matrix component. SS RBCs were preincubated with Yoda1 (0.1–10 μ M; 30 min) and their rolling along and adhesion to laminin (LN) was monitored under conditions of shear stress using a microfluidic flow assay (3 min, 37°C, 0.25 dyn/cm², as described in Section 2). Piezo1 stimulation augmented the rolling of SS RBCs and their adhesion to LN-coated microchannels, at the concentration of 0.5 μ M Yoda1 (Figure 1F,G; Figure S2C shows representative photographs of rolling and adhered RBCs). In contrast, both the basal rolling and adhesion of AA RBCs to LN was significantly lower than those of SS RBCs (Figure S2A,B), and incubation of AA RBC with 0.5 μ M

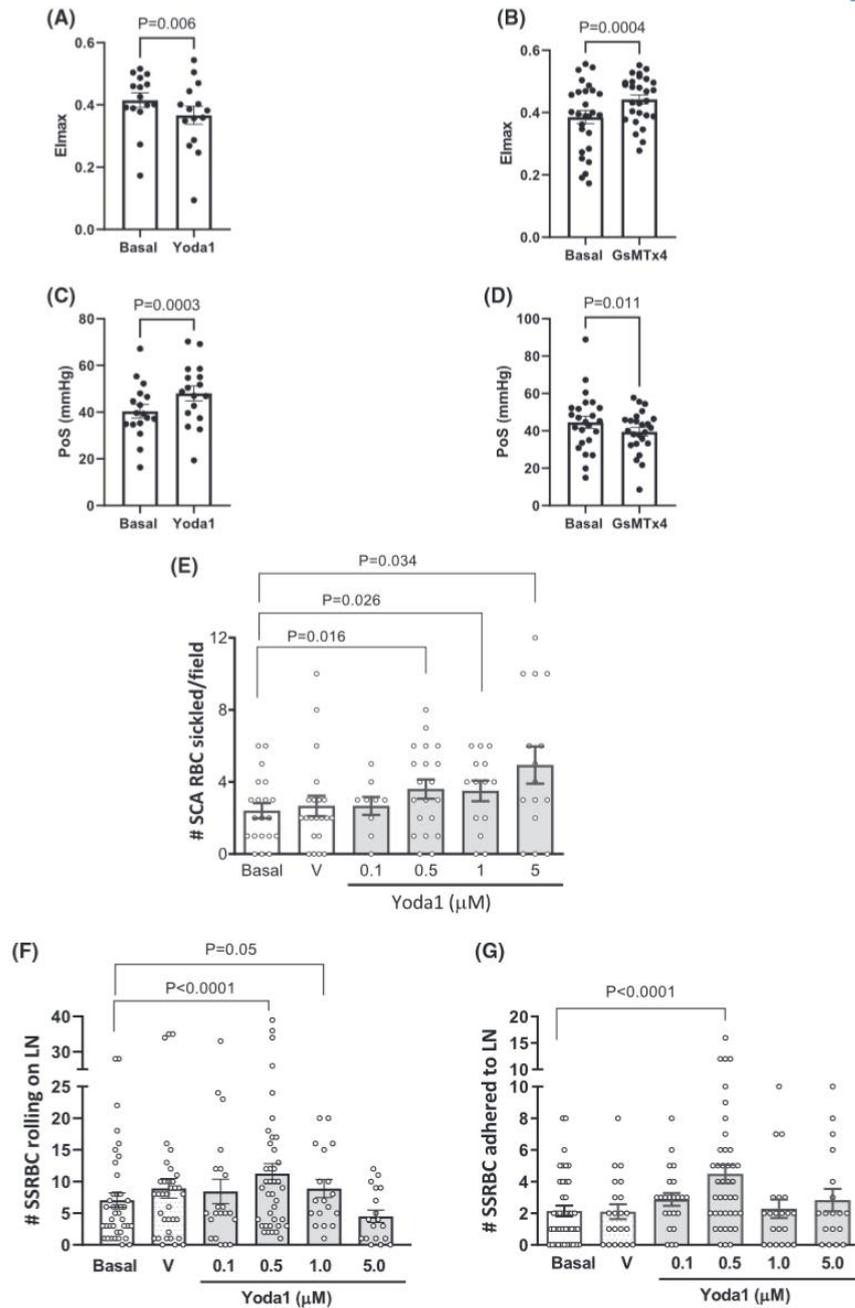


FIGURE 1 Effects of PIEZO1 channel activation and inhibition on sickle RBC deformability, sickling and adhesive properties. (A–D) Oxygenscan ektacytometry was used to measure EImax (maximal RBC deformability in normoxia) and oxygen pressure at which RBCs start to become rigid and change shape (point of sickling, PoS), in response to Piezo1 activation or inhibition. SS RBC were incubated for 30 min with Yoda1 (10 μM , $n=15, 17$; A and C), or GsMTx4 (10 μM , $n=27, 24$; B and D) (37°C) before measurement of RBC deformability in normoxia (A and B, respectively) and PoS during progressive deoxygenation (C and D, respectively). p values calculated by paired t -test. (E) Sickling of SS RBC after incubation with DMSO vehicle or Yoda1 (0.1–10 μM , 30 min), observed by light microscopy ($n=3$ –7 patients; three fields per channel). (F–G) Rolling and adhesion of sickle RBCs on laminin (LN)-coated microchannels using a microfluidic platform, under shear stress of 0.25 dyn/cm^2 (400 μm -wide microchannel; 4 $\mu\text{g}/\text{mL}$ LN, 37°C, 2 min) ($n=6$ –12 patients per treatment; three fields per channel). Data are expressed as the number of RBCs observed rolling or adhering per 0.186 μm^2 area. V, DMSO vehicle. For (E–G), p values were calculated by Mixed effects analysis with Sidak's multiple comparison test.

Yoda1 did not significantly increase their rolling or adhesion (Figure S2A,B; $p > 0.05$).

Flow cytometry showed that, while SS RBCs highly express the basal cell adhesion molecule (BCAM; CD239) on their membrane surface (Figure 2A),³⁴ the activation of Piezo1 by Yoda1 (0.5–5.0 μM ; 30 min) did not significantly alter cell surface expression of this laminin-binding molecule (Figure 2C). Expression of the CD49d subunit of the VLA-4 integrin (an adhesion molecule that binds primarily to fibronectin or VCAM-1) is also increased on SS RBCs, compared to RBC from healthy controls; however, activation of Piezo1 on SS RBCs by Yoda1 (0.5–5.0 μM) did not significantly alter VLA-4 cell surface expression (Figure 2B,D).

Importantly, co-incubation of the SS RBCs with an antibody that neutralizes the binding activity of BCAM completely abolished the effect of Yoda1 (0.1 and 0.5 μM ; 30 min) on SS RBC rolling and adhesion, while an antibody that neutralizes CD49d binding had no effect on these parameters (Figure 2E,F). Data indicate that Piezo1 activation induces SS RBCs adhesion via a mechanism that increases the binding affinity of BCAM, rather than by BCAM expression.

Piezo1 stimulation triggers pronounced membrane hyperpolarization linked to Gárdos Channel activation

Membrane potential measurement showed that Piezo1 stimulation triggers immediate and pronounced membrane hyperpolarization of SS RBCs, suggestive of Gárdos channel activation triggered by Ca^{2+} entry, via a phenomenon that was exacerbated by low oxygen partial pressure (Figure 3A–C). Hyperpolarization was significantly more pronounced under low oxygen partial pressure conditions (Figure 3B) and this hyperpolarization lasted longer (Figure 3C). Consistent with this result, flow cytometry demonstrated a clear effect of Piezo1 activation and inhibition by Yoda1 (10 μM ; Figure 3D) and GsMTx4 (10 μM ; Figure 3E) on Ca^{2+} influx. Yoda1 also increased Ca^{2+} in AA RBC ($n=8$) (MFI: 221 ± 10 vs. 251 ± 6 ; $p < 0.01$), but no significant effect of GsMTx4 was observed on AA RBC (231 ± 9). Further experiments showed Ca^{2+} influx was directly associated with the propensity of sickling under decreased O_2 partial pressure (Figure 3F) and subsequent Gárdos channel activation, as demonstrated by co-incubation of SS RBCs with TRAM34, a Gárdos channel inhibitor, albeit no direct measurement of calcium influx kinetics has been conducted in this series of experiments. This Ca^{2+} entry through Piezo1 is sufficient to activate the Gárdos channel as shown by the transient hyperpolarization recorded, where the extent of hyperpolarization triggered by calcium through Piezo1 activation, both in oxy and deoxy conditions, correlated with the susceptibility of RBCs to sickle, as measured by the PoS by oxygenscan (Figure 3G,H).

In additional experiments, SS RBC suspensions were incubated with the cell permeable Ca^{2+} chelator, BAPTA-AM (10 μM , 30 min), before observing Yoda1-induced cellular rolling and adhesion to LM-coated microchannels under shear stress. The chelation of intracellular Ca^{2+} abrogated the enhanced cell adhesion triggered by Piezo1 activation using Yoda1 (0.1 and 0.5 μM), but did not affect cell rolling on laminin (Figure 3I,J).

Piezo1 stimulation induces PS exposure and CD47 expression on sickle RBC

Flow cytometry experiments demonstrated a clear effect of Piezo1 activation by Yoda1 (10 μM) on CD47 expression and on PS exposure on SS RBCs (Figure S3A,B), where CD47 expression was suppressed and PS exposure was augmented. The use of GsMTx4 (10 μM) increased CD47 exposure but had no impact on PS exposure, compared to the control condition (Figure S3C,D). The percentage of RBCs with externalized PS was higher in SS than in AA ($n=8$) (0.28 ± 0.16 ; $p < 0.001$) and Yoda1 tended to increase PS exposure in AA RBCs (0.35 ± 0.17 ; $p = 0.08$). GsMTx4 had no effect on PS exposure in AA RBCs (0.27 ± 0.13). CD47 exposure in AA RBCs (6492 ± 2014) was higher than in SS RBCs ($p < 0.001$). Neither Yoda1, nor GsMTx4 significantly affected CD47 exposure on AA RBCs (6667 ± 2187 and 7476 ± 2594 , respectively).

Sickling tendency and RBC PS exposure are greater in patients with the gain of function E756del variant in PIEZO1, compared to those without

Using next generation sequencing, eight frequent SNV were found in SCA patients (rs2911442 [13%], rs7404939 [20%], rs11076706 [23%], rs13333358 [34%], rs35265318 [12%], rs59446030 [13%], rs750038587 [18%], rs1803382 [17%]) in the heterozygous or homozygous state. Of interest, the PoS and the percentages of RBCs with externalized PS were significantly higher in patients with rs59446030 (E756del) in the heterozygous ($N=7$) and homozygous states ($N=3$). PoS was 42.3 ± 7.2 and 48.1 ± 6.8 mmHg in SCA patients without and with the gain of function E756del variant in Piezo1, respectively ($p < 0.05$); PS = 2.4 ± 1.8 and $4.2 \pm 2.5\%$ of positive RBCs in SCA patients without and with the gain of function E756del variant in Piezo1, respectively ($p < 0.01$). The presence of the other frequent SNV had no effect on RBC rheological properties and biological parameters (data not shown).

DISCUSSION

Piezo1 is a non-selective membrane cation channel, involved in the regulation of RBC hydration status and volume

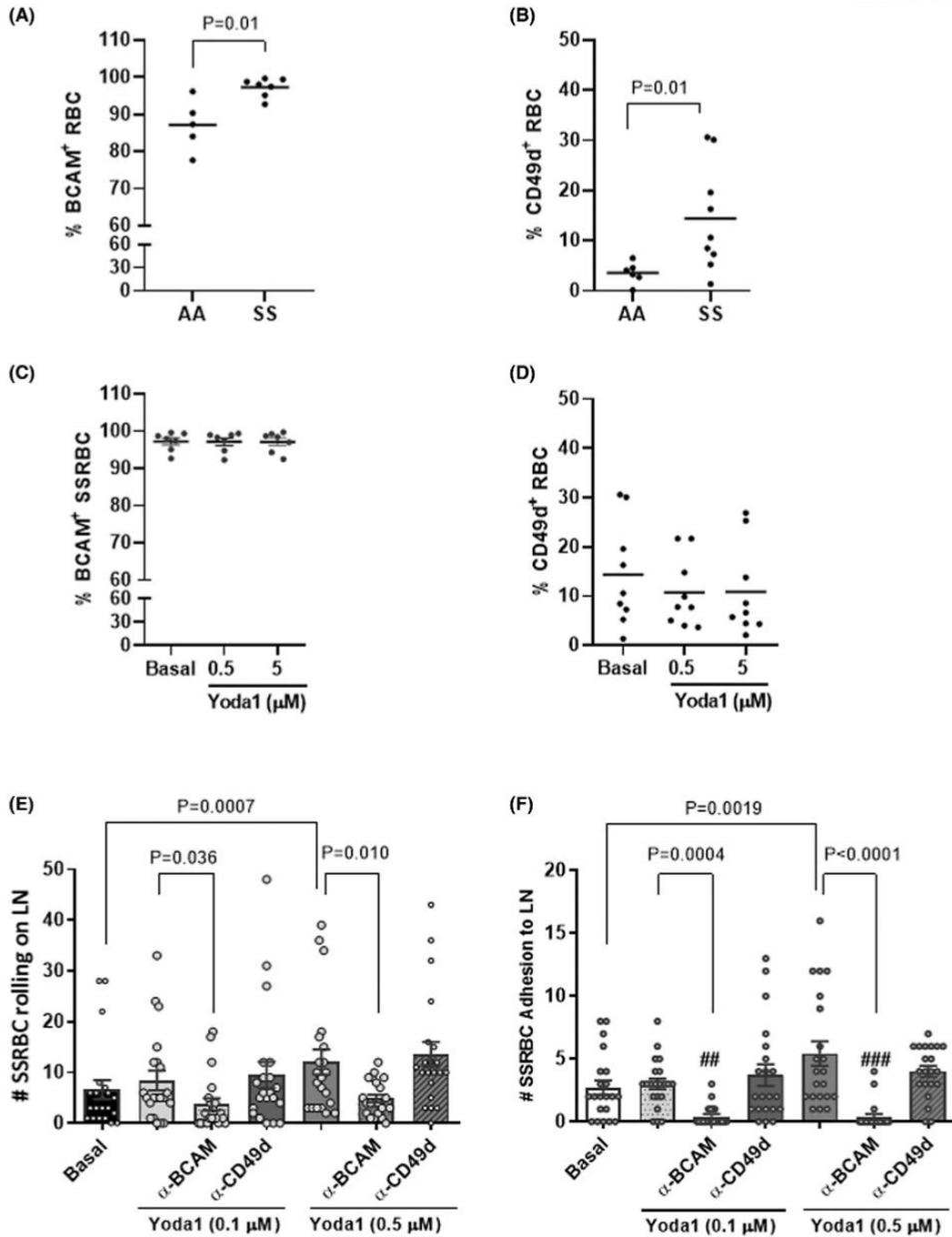


FIGURE 2 Piezo1-mediated activation of sickle red blood cell rolling and adhesion on laminin occurs via a BCAM-dependent mechanism. (A, B) The surface expressions of (A) BCAM (CD239) or (B) CD49d molecules were determined by flow cytometry on 50 000 gated RBC from healthy control (AA; $n=5$) or SCA (SS; $n=7$ and 9 , respectively) individuals. In (C, D), SS RBC were co-incubated with Yoda1 (30 min) before determining the cell surface expressions of (C) BCAM and (D) CD49d by flow cytometry. $n=7$ (C) and 9 (D). (E, F) SS RBC ($n=7$) were co-incubated with Yoda1 and antibodies that neutralize the functions of the BCAM or CD49d adhesion molecules, and RBC rolling (E) and adhesion (F) to laminin (LN) were evaluated using the microfluidic assay (400 μm -wide microchannel; 4 $\mu\text{g}/\text{mL}$ LN; 0.25 dyn/cm^2 , 37°C, 3 min; ≤ 3 fields per channel/patient). Non-parametric ANOVA, Dunn's multiple comparison test.

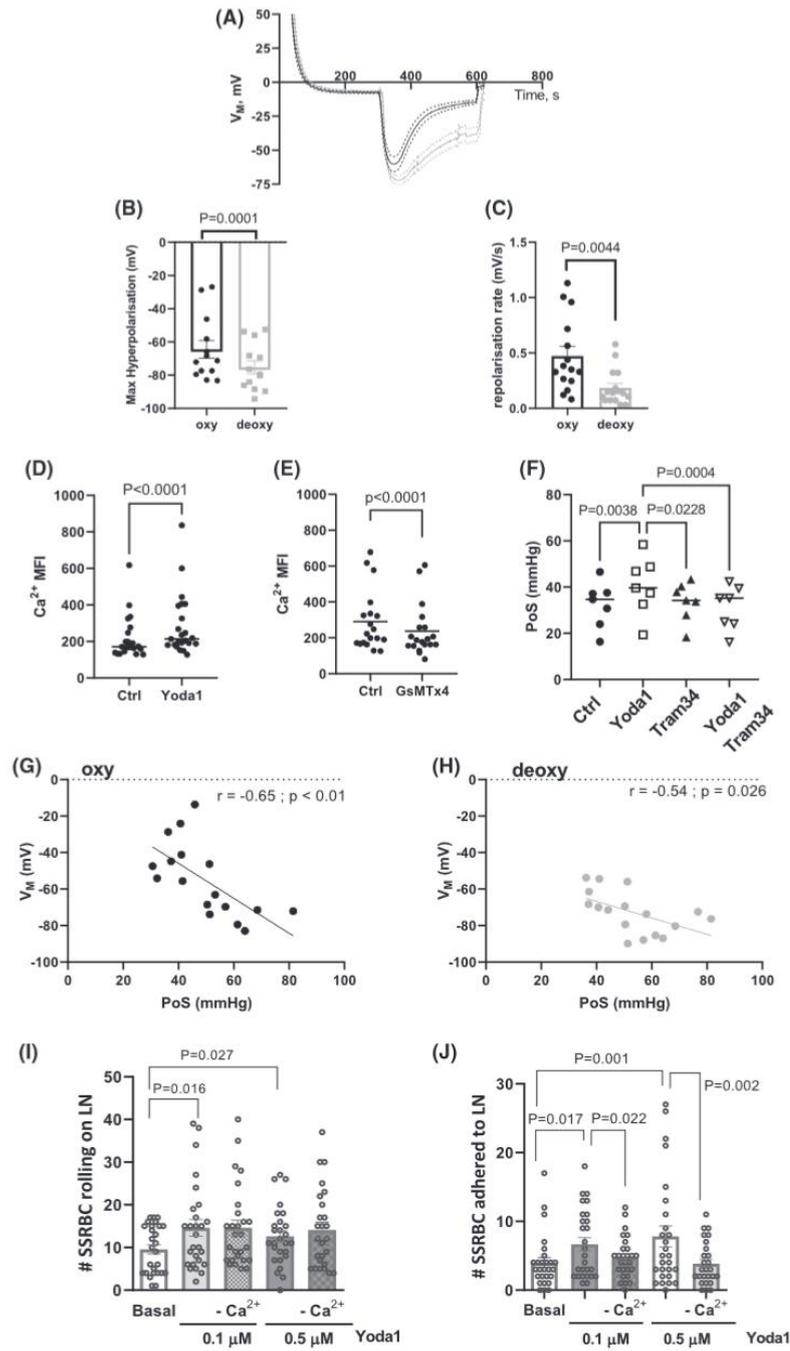
under mechanical constraints.³⁵ Hereditary xerocytosis, a rare haemolytic anaemia, is demonstrated to be due to gain of function mutations in PIEZO1.³⁶ Several electrophysiological studies demonstrated that this gain of function phenotype is characterized by a delayed inactivation of Piezo1, accompanied by increased cation permeability.^{10,37,38} Vandorpe et al.³⁹ previously demonstrated that hypoxia activated a Ca^{2+} -permeable cation conductance, which was sensitive to GsMTx-4, a mechanosensitive and stretch-activated ion channel inhibitor, in human and mouse sickle RBCs. Indeed, Brugnara⁸ suggested that P_{sickle} , that is, the increase in permeability to Ca^{2+} and other ions occurring during deoxygenation, and sickling, which causes RBCs from SCA patients to become so dehydrated, could be mediated by Piezo1.

Our findings showed that activation of Piezo1 in sickle RBCs, but also in RBCs from healthy individuals, leads to intracellular Ca^{2+} accumulation, while its inhibition caused the opposite in sickle RBCs. Although we did not examine the detailed kinetics of intracellular Ca^{2+} changes using Yoda1 and GSMTx4, our results reinforce the previous findings of Wadud and colleagues.¹⁷ Consequently, the stimulation of Piezo1 caused immediate pronounced membrane hyperpolarization of sickle RBCs, linked to Gárdos channel activation, via a phenomenon that was exacerbated by low oxygen partial pressure. This channel activation, and possibly ensuing RBC dehydration, affected the deformability of RBCs and increased their propensity to sickle when oxygen pressure was reduced. Experiments conducted in AA RBCs also demonstrated that activation of Piezo1 was followed by a decrease in their deformability. Electrophysiological and ektacytometry measurements were also both performed in the same subset of SCA patients and showed significant negative correlations between the degree of RBC membrane hyperpolarization and the PoS under Yoda1 stimulation. A comparison of SCA patients with the gain of function variant in Piezo1 (E756del), to those without, demonstrated a propensity of RBCs to sickle at higher oxygen partial pressure (greater PoS) in the former population, confirming previous findings showing an association between the E756del allele and RBC density in SCA.⁴⁰ The use of a Gárdos channel inhibitor (i.e. TRAM34) with the Piezo1 activator (Yoda1) during oxygen gradient ektacytometry measurements restored baseline PoS, indicating that the increased propensity of RBC to sickle during deoxygenation and upon Piezo1 activation was mediated ultimately by Gárdos channel

activation. Altogether, these findings support a key role of Piezo1 protein in RBC sickling in SCA that could be mediated by RBC dehydration (although not directly tested in the present study) following Gárdos channel activation. We suspect that alterations in membrane tension and red cell morphology may further activate the mechanosensitive Piezo1 channel, leading to a vicious cycle of cellular dehydration, HbS polymerization and, thereby, cell sickling. Our results also showed that sickle RBCs responded to GsMTx4 without prior stimulation with Yoda1. These findings could indicate that Piezo1 is constitutively active in sickle RBCs and could contribute to their higher nominal calcium concentration compared to healthy RBCs. However, it is worth noting that almost all experiments performed in this study were done under flow/shear stress conditions that could mimic the mechanical stress applied to Piezo1 within the circulation and activate the channel. Furthermore, wall shear stress has been shown to be increased in SCA patients, which could further increase the mechanical constraints that RBCs are subjected to, further increasing Piezo1 activation and subsequent deleterious consequences.⁴¹

The stimulation of Piezo1 also caused a rise in the number of RBCs positive for PS and a decrease in membrane CD47 level; two changes indicating enhanced RBC senescence. These findings are consistent with a recent study showing that Yoda1 caused an increase in intracellular Ca^{2+} and PS exposure on sickle RBCs, which was inhibited by its antagonist Dooku1 and the Piezo1 inhibitor GsMTx4.¹⁷ Accumulation of Ca^{2+} in RBCs may activate scramblase, causing the disruption of membrane phospholipid asymmetry and the externalization of PS at the outer leaflet of the RBC membrane.^{42,43} Additionally, Wadud et al.¹⁷ showed that Yoda1 could induce PS externalization independently of extracellular calcium concentration, suggesting an effect that is not mediated by Piezo1-dependent calcium influx. It should also be mentioned that Yoda1 and GsMTx4 do not exhibit complete specificity towards Piezo1, and may elicit off-target effects, implying that other channels or pathways could be involved in the effects observed. Increased PS externalization and decreased membrane CD47 level have been reported in sickle RBCs,⁴⁴ compared to healthy RBCs, indicating accelerated RBC senescence in this disease,^{45,46} which may lead to early recognition by macrophages and increased erythrophagocytosis.^{46–48} Interestingly, SCA patients with the

FIGURE 3 Effects of Piezo1 activation on membrane hyperpolarization, sickling and adhesion are mediated by direct activation of the Gárdos channel or calcium influx. Changes in membrane potential of SS RBCs (A) following Piezo1 activation by YODA1 (625 nM, $n = 13$ averaged curve \pm SEM) in oxy (black) and deoxy (grey) conditions, showing a more pronounced hyperpolarization (B), associated with a decreased pace of repolarization (C) when partial pressure of O_2 is reduced to 10% of nominal air partial pressure (0.8 mg/mL, $n = 13$). (D–E) Calcium influx in SS RBCs following Yoda1 (10 μM) or GsMTx4 (10 μM) incubation was determined by flow cytometry in 50 000 gated RBCs ($n = 23, 19$). (F) Oxygen gradient ektacytometry was used to observe the effect of inhibition of the Gárdos channel with Tram 34 (10 μM) on the point of sickling of SS RBCs incubated with/without Yoda1 (10 μM ; 30 min, 37°C, $n = 7$). (G, H) Correlations between RBC membrane hyperpolarization following Piezo1 activation and PoS, both in oxy (G) and deoxy conditions (H). (I, J) Calcium chelation of SS RBCs with BAPTA-AM (10 μM , 30 min) did not significantly affect the Yoda1-induced rolling of cells on laminin (LN) (H), but significantly decreased Yoda1-induced SS RBC adhesion (I), as demonstrated by a microfluidic assay (400 μm -wide microchannel; 4 $\mu\text{g}/\text{mL}$ LN; 0.25 dyn/cm^2 , 37°C, 3 min; $n = 9$ patients per group, ≤ 3 fields per channel). Data are expressed as the number of RBC observed per 0.186 μm^2 area. Paired *t*-test, except (F), Friedman multiple comparison test and (I, J), non-parametric ANOVA/Sidak's multiple comparison test.



gain of function variant in Piezo1 (E756del) had a greater number of RBCs positive for PS than those without. The membrane phospholipid asymmetry disruption and the resulting membrane blebbing could also favour the release of extracellular vesicles from RBCs, which are known

to play a key role in SCD pathophysiology and vascular dysfunction.^{45,49}

The present study also explores the effect of Piezo1 activation on RBC adhesion. We found that activating Piezo1 resulted in increased rolling and adhesion of sickle RBCs

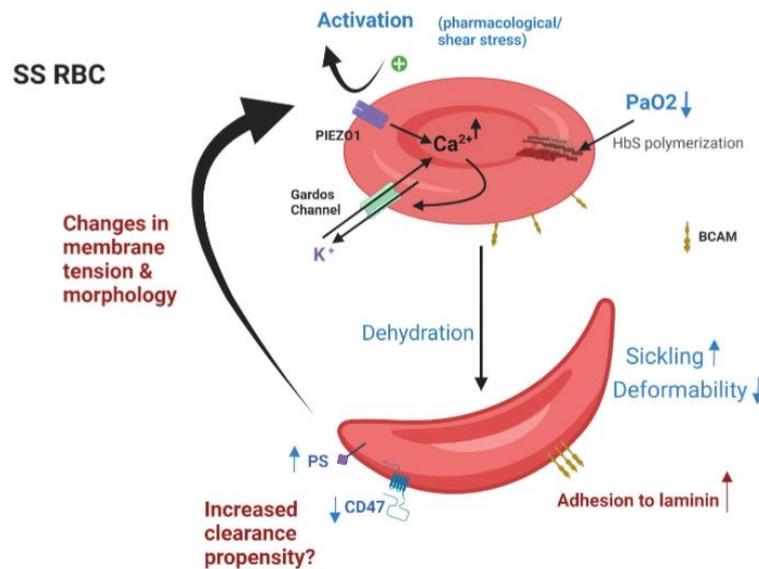


FIGURE 4 Role of Piezo1 in modulating red blood cell (RBC) physiology and function in sickle cell anaemia (SCA). Activation (pharmacological or physical) of Piezo1 on sickle RBCs leads to intracellular calcium influx and probable Gardos channel activation, with triggering of further calcium influx and potassium efflux, leading to RBC dehydration. These events exacerbate the increased sickling of sickle RBCs and reduced deformability that occurs under lower oxygen pressure (PaO_2). Piezo1 activation also leads to phosphatidylserine (PS) exposure on the RBC surface, possibly facilitating microvesicle production, and reduced expression of CD47, potentially augmenting cell clearance propensity. Furthermore, Piezo1 activation induced the adhesion of sickle RBC to laminin via a mechanism dependent upon BCAM adhesion molecule activation (conceivably mediated by BCAM clustering). Increased sickle RBC adhesiveness can contribute to vaso-occlusive processes. Figure created at [Biorender.com](https://www.biorender.com).

to laminin, without any changes in BCAM expression at the RBC membrane level. Increased expression of the Lutheran(Lu)/BCAM glycoproteins, which bind to the laminin $\alpha 5$ chain, has been reported on sickle RBCs and a role for this molecule in the increased adhesive properties of sickle RBCs suggested.^{50,51} Furthermore, a more recent study reported that Gardos channel activation on RBCs triggers cellular senescence and the shedding of glycophorin-C-containing vesicles, resulting in Lu/BCAM and CD44 activation.⁵² Importantly, the use of an antibody to neutralize the function of the BCAM molecule decreased the adhesion of Piezo1-stimulated sickle RBCs to laminin, while an antibody that binds to the CD49 integrin subunit, which is also highly expressed on sickle RBCs, had no effect on sickle RBCs adhesion. Although we did not directly measure the effects of BATPA-AM on RBC Ca^{2+} in the present study, our findings suggest that BCAM-dependent adhesion of Piezo1-activated sickle RBCs could also be dependent on intracellular Ca^{2+} accumulation. As Piezo1 stimulation by Yoda1 was not associated with any alteration in RBC surface BCAM expression, we suggest that the increase in the adhesion of SS RBCs to laminin was mediated by BCAM clustering at the cell surface. Indeed, Ca^{2+} -dependent BCAM clustering and consequent adhesion to laminin has recently been described in uninfected RBCs during malaria infection⁵³ and on sickle RBCs under conditions of oxidative stress.⁵⁴ One curiosity of our findings was that we found that activation of SS RBC

adhesion to laminin occurred at lower concentrations of Yoda1 than needed to reduce cell deformability and augment sickling. A range of Yoda1 concentrations have been used to activate Piezo1, with recent studies employing between 1 and $10 \mu\text{M}$ Yoda1 to induce PS exposure on SS RBCs¹⁷ and $1 \mu\text{M}$ to stimulate integrin-mediated erythroblast adhesive interactions.⁵⁵ We postulate that the exacerbated sickling of sickle RBCs that we observed at concentrations of greater or equal to $5 \mu\text{M}$ Yoda1 may in fact interfere with the ability of the cells to adhere to ligands such as laminin. Notably, it is known that Piezo1 sensitivity to shear stress is dependent on the surrounding lipid environment, and also on the proteins of the extracellular matrix with which Piezo1 channel can interact.^{56,57} Clearly, the topology and the Piezo1-protein interaction at the RBCs membrane is still elusive, but the preferential location of Piezo1 channels at the rim of healthy red blood cells⁵⁸ suggests that, in the case of aberrant forms (such as upon sickling), this location may be altered and induce different mechanisms or sensitivities to shear stress.

In conclusion, our results demonstrated that Piezo1 activation and the resulting Ca^{2+} accumulation provoke a decrease in sickle RBC deformability and a greater propensity of RBCs to sickle upon deoxygenation via its effects on Gardos channel activation. In addition, Piezo1 activation increased sickle RBC senescence and RBC adhesiveness in a calcium- and BCAM-dependent manner (Figure 4). Our results strongly support a role of the Piezo1 mechanosensor

in the alterations in RBC properties that can contribute to vaso-occlusive crises and other complications in SCA and may represent a potential therapeutic target molecule for the treatment of this devastating disease. Nevertheless, our results showed a high variability for most of the parameters studied that could reflect the fact that RBCs from SCA patients are very heterogeneous in properties. Further studies are warranted in larger groups of patients to test the associations between clinical severity, membrane electrophysiological properties, RBC adhesiveness and sickling propensity.

AUTHOR CONTRIBUTIONS

E.N., N.C., S.E. and P.C. designed the study, analysed data and wrote the manuscript. F.C.L. and N.C. performed adhesion experiments. C.B., E.N., P.L.B., R.C. and S.E. performed rheological and flow cytometry experiments. A.H. and S.E. carried out membrane potential measurements. C.R., P.J., J.B. and C.G. did N.G.S. protocols. A.G., S.P., Y.V., F.F.C. and S.T.O.S. supervised patient diagnosis and management (Lyon and UNICAMP centres). P.L.B. performed flow cytometry. All authors read and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors report no conflicts of interest relevant to this study.

DATA AVAILABILITY STATEMENT

All raw data are available, upon reasonable request, from the corresponding authors.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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7.5 Study n°5

Putative adverse effect of Δ^9 THC on sickle red blood cells

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As previously introduced for Piezo1, the rise in cation permeability, especially of Ca^{2+} , promotes RBC dehydration through Gárdos channel activation. This increased permeability is named P_{Sickle} but the protein involved has not been clearly identified. Recently, TRPV2 has been found in RBCs. TRPV2 is a cation ion channel which presents similar characteristics to Piezo1, such as mechanosensitivity as well as a strong permeability for Ca^{2+} .

It can be activated by cannabinoid compounds such as $\Delta^9\text{THC}$ and CBD. TRPV2 belongs to the family of mechanosensitive channels, making it a potential candidate for P_{Sickle} .

The aim of this study was to assess the presence of TRPV2 channels on sickle RBCs and to investigate its role on Ca^{2+} influx and membrane hyperpolarization in normoxia and hypoxia. We compared the results to those from healthy RBCs. Flow cytometry was used to get a semi-quantitative measurement of Ca^{2+} influx. The membrane potential variations were measured after $\Delta^9\text{THC}$ exposure in oxygenated and deoxygenated conditions, using the MBE method. Furthermore, the changes in membrane currents elicited by $\Delta^9\text{THC}$ were detected by the Patch-Clamp technique. Flow cytometry results showed that sickle RBCs responded to $\Delta^9\text{THC}$ more than control samples. The MBE method revealed that $\Delta^9\text{THC}$ had an effect only on sickle RBCs, triggering a hyperpolarization linked to Gárdos channel activation. Interestingly, the phenomenon was exacerbated in deoxygenated conditions. The

hyperpolarization was abolished upon Gárdos channel inhibition, which indicates that the activation of the TRPV2 channel can lead to a Gárdos effect. Patch-Clamp recordings displayed higher currents and a higher number of responding cells in SCA patients than in healthy controls. The results clearly indicated that the activation of TRPV2 in sickle RBCs leads to a sufficient Ca^{2+} influx that eventually trigger Gárdos activation and may contribute to the dehydration of SCA RBCs. The activation of TRPV2 channel was accompanied by a decrease, even if slight, of RBC deformability. Our results suggest that TRPV2, like Piezo1, could be involved in the P_{Sickle} pathway.

Putative adverse effects of Δ^9 THC on sickle red blood cells

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To the Editor:

Sickle Cell Disease (SCD) is characterized by the presence of an abnormal hemoglobin HbS that crystalizes in deoxygenated conditions, causing the sickling of red blood cells (RBCs). During deoxygenation, increased RBC cation conductance, especially of Ca^{2+} , has been shown to promote RBC dehydration through Gárdos channel activation, hence increasing RBC sickling. Thus, any pathway that may increase Ca^{2+} represents a threat to the lifespan of the sickle RBCs within the circulation. Notably, Piezo1 has been recently demonstrated to play a role in the increased Ca^{2+} entry observed in sickle RBCs¹. Furthermore, the presence of the transient receptor potential channel vanilloid type 2 (TRPV2) in RBCs has also been validated², opening immediate questions on its physiological relevance for RBC life. TRPV2 channels share several features with Piezo1 such as mechanosensitivity as well as a strong permeability for calcium ions³. More importantly, it can be activated by Δ^9 -tetrahydrocannabinol (Δ^9 THC) even in RBCs⁴. The instantaneous changes in cell shape and filterability that occur upon Δ^9 THC stimulation in normal RBCs⁴ have led us to wonder what could happen to sickle RBCs, which are even more susceptible to alterations of shape and deformability.

Figure 1A shows whole-cell current traces of normal (AA) and sickle (SS) RBCs before and after stimulation with 80 μM Δ^9 THC. While AA RBCs display a conductance below 0.5 nS (Figure 1A-left panel, B)⁵, SS RBCs exhibit a three times higher conductance as already demonstrated in other SCD studies (Figure 1A, B)⁶. Furthermore, despite both cell types respond positively to Δ^9 THC, the magnitude of the response obtained for SS RBCs is much more pronounced with an average conductance of 2.3 ± 0.5 nS (n=10) compared to AA cells (0.7 ± 0.2 nS, n=4). Importantly, not all cells tested in both

groups respond to Δ^9 THC stimulation, showing again the heterogeneity of the RBC population⁴. Nevertheless, the percentage of responding RBCs in the SS group is much higher than the percentage observed in the control cells (20.0% vs. 46.7% n=4 out of 20 and n=10 out of 21 respectively) (Figure 1A). When monitored by the Macey-Bennekou-Egee (MBE) method, the addition of Δ^9 THC causes only a very small change in membrane potential of AA RBCs (-3.75 ± 0.7 mV, n=12, Figure 1C, D) whereas the same concentration of Δ^9 THC triggers a highly significant hyperpolarization of -16.3 ± 1.5 mV in SS RBCs (n=35, p<0.001, Figure 1C, D). This hyperpolarization is strictly dependent on oxygen, as once the oxygen tension is reduced to 10% of the atmospheric partial oxygen pressure, the hyperpolarization reaches -28.4 ± 2.3 mV (n=20, Figure 1C, D). The observed hyperpolarizations are accounted for an increased activity of the Gárdos channel since all are prevented by pre-incubation with charybdotoxin (100 nM, Figure 1D). It is noteworthy that the resting membrane potential of sickle RBCs is on average less electronegative than AA cells (Figure 1E) and that such a difference may be related to a different ageing process (120 days vs. 15-20 days, respectively).

The possibility that the copy number of TRPV2 channels in SS RBCs is higher than in control cells was tested by western blot on two different controls and three patients (Figure 1F). No difference in expression could be detected between the two populations, indicating that the differences in response between the two populations are likely to be related to the differential ability of TRPV2 to drive Ca^{2+} entry. The Gárdos channel is strictly dependent on the intracellular Ca^{2+} concentration, which affects not only its opening but also its open probability⁷. Therefore, it was necessary to determine whether the Ca^{2+} movements were similar between the two cell populations. To this end, the Ca^{2+} content in RBCs after stimulation with Δ^9 THC was assessed by flow cytometry using Fluo-4 fluorescence intensity. As expected, SS RBCs have a higher Ca^{2+} content than AA cells at the resting state (Figure 1G, H). Thus, the fluorescence intensity is half an order of magnitude higher for SS compared to AA RBCs (Figure 1G, grey and black traces respectively). Moreover, when the cells are subjected to Δ^9 THC stimulation, not only the number of responding RBCs is higher for SS, but so is the intensity of the measured fluorescence (Figure 1G, purple and red traces & Figure 1H). Importantly, it should be noted that this increase in $[\text{Ca}^{2+}]_i$ affects more the SS cells compared to the AA RBCs since only 25% are positive for high Ca^{2+} content compared to almost 50% of SS RBCs (Figure 1H). This finding is consistent with the results obtained by the patch-clamp technique (Figure 1A, B) or the MBE method (Figure 1C). Indeed, only 20% of the control RBCs were responsive to Δ^9 THC compared to 46.7% of the SS RBCs in the whole-cell configuration of the patch-clamp technique. More importantly, the MBE method measures the average change in membrane potential of a whole population of cells. The response obtained is affected by the capacity of all the population to react simultaneously to the stimulus. It is therefore likely that the large difference observed between the two populations (AA vs. SS) is also partly due to the susceptibility of all or part of the cell population to respond to Δ^9 THC. Finally, Gárdos channel activity, measured by the extent of hyperpolarization, is dependent on other conductance pathways in the cell, in

particular chloride, which counteracts the efflux of K^+ (from an electrical point of view), but also on the capacity of the cell to limit the intracellular Ca^{2+} increase by the action of the Ca^{2+} -pump (PMCA). This also explains the slow repolarization observed in SS RBCs. To investigate the potential effects of Δ^9 THC on RBC deformability, ektacytometry tests were practiced. The results revealed a significant loss of SS deformability (Figure 1I) in response to Δ^9 THC without any effect on normal AA cells (data not shown).

From all presented data, we can conclude that:

1. RBCs from SCD patients are more responsive to acute Δ^9 THC stimulation compared to control RBCs without any difference in the copy number of TRPV2 channels. The immediate consequences of this reactivity to Δ^9 THC are a rapid and prolonged increase in intracellular Ca^{2+} concentration, which – in contrast to healthy RBCs – reaches and exceeds the threshold for activation of the Gárdos channel. The latter stays open long enough to presumably cause a substantial loss of potassium, overcompensating the volume increase caused by the sodium entry through TRPV2 and thus the propensity to sickle by increasing the intracellular hemoglobin concentration.
2. The heterogeneity observed in Δ^9 THC responses clearly raises the question of the relationship between RBCs ion channel response and the age of the cells within the circulation. The physiological environment of sickle RBCs is disturbed by increased oxidative stress, coupled with nitric oxide and ions accumulation. This likely could change the activity of various channels, which makes it interesting for future investigations.

These results raise a further question in the perspective of recent and very encouraging results on the use of cannabinoid derivatives (CBD) for the treatment of pain associated with the disease⁸ as well as studies showing that daily cannabis users with SCD had worse pain episode severity scores than others, associated to a lower rate of hospital admission and emergency room⁹.

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Conflict of interest statement

The authors declare no conflict of interest.

Data availability statement

Requests for data should be sent to the corresponding author at egee@sb-roscoff.fr

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Ethics approval

Blood from healthy volunteers was withdrawn upon written informed consent (EFS, Etablissement Français du Sang), in accordance with the guidelines of the Helsinki declaration of 1975, as revised in 2008. This work has been approved by the institutional (CNRS) Ethical committee and by the French Ministry of Research (declaration DC-2019-3842).

Patients (HbSS genotype) were recruited, with informed consent, from Hospices Civils de Lyon and Institut d'Hématologie et d'Oncologie Pédiatrique de Lyon, France. All subjects were in clinical steady-state at the time of the study; that is, without experiencing any vaso-occlusive crisis or other acute medical complication within the last 2 months, and without any blood transfusions for at least 3 months before inclusion. The study was conducted in accordance with the guidelines set by the Declaration of Helsinki and approved by the Regional Ethics Committees (L14-127, Université de Lyon; 4.649.745).

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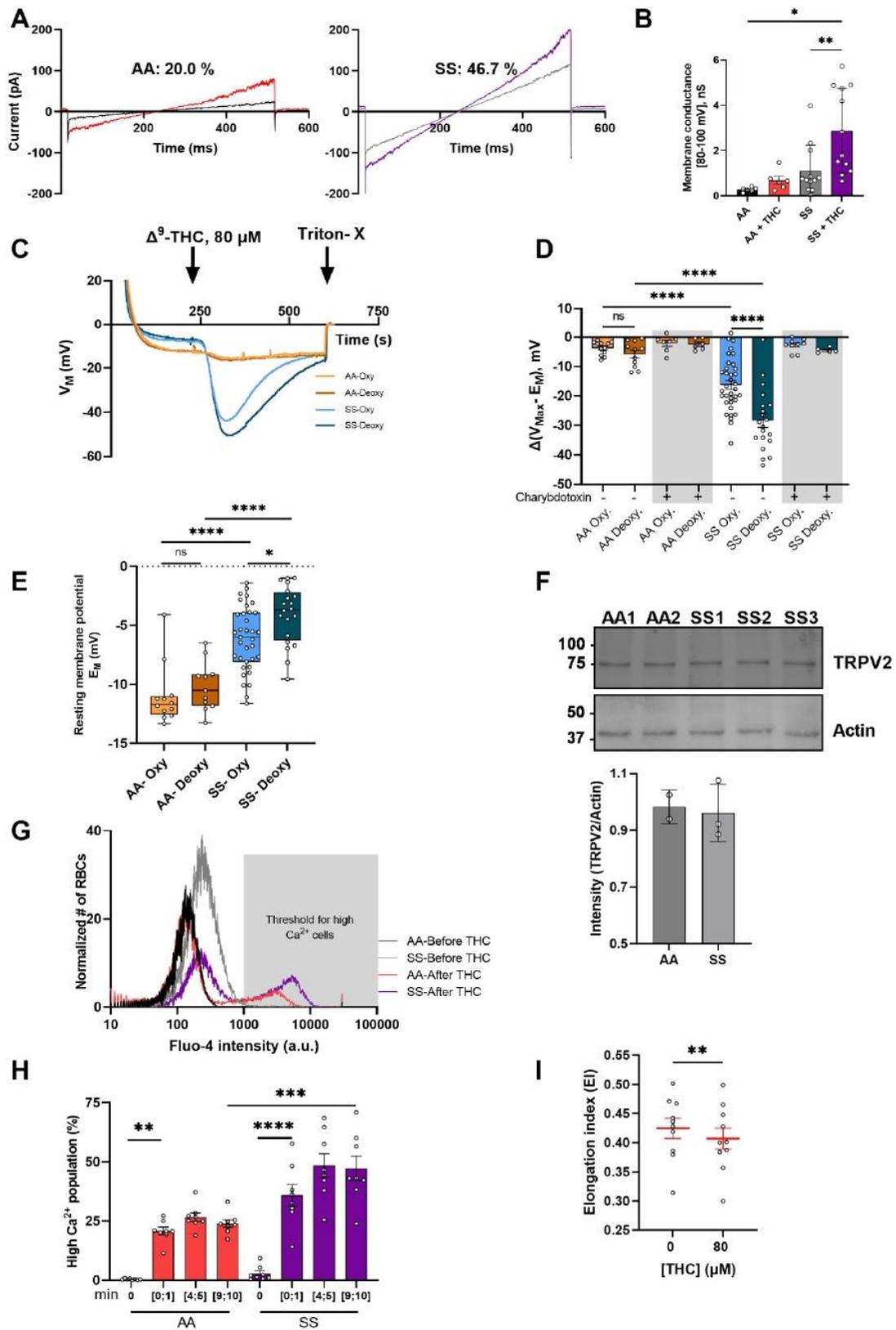


Figure 1: TRPV2 activation in SS and AA RBCs. (A) Raw traces of current amplitude (pA) were recorded using patch-clamp from healthy controls (AA cells), n=4 (left panel), before Δ^9 THC (black trace) and after 80 μ M of Δ^9 THC (red trace), and from sickle patients (SS cells), n=10 (right panel), before Δ^9 THC (grey trace) and after 80 μ M of Δ^9 THC (purple trace). The percentage of responding cells is indicated for each type of cells in both panels of Figure 1A. (B) Differences in membrane conductance (nS) before and after Δ^9 THC application, calculated at positive voltages (between 80 and 100 mV) in AA and SS cells (paired t-test, **p < 0.01). (C) Representative traces for the variations of membrane potential after injection of Δ^9 THC (80 μ M) in AA oxygenated cells (orange trace), AA deoxygenated cells (brown trace), SS oxygenated cells (light blue trace), and SS deoxygenated cells (dark blue trace). (D) Statistical comparison of the hyperpolarization magnitudes reached at different conditions with and without inhibiting the Gárdos channel with charybdotoxin. N=12 for AA oxygenated cells, N=11 for AA deoxygenated cells, N=7 for AA oxygenated cells with charybdotoxin and N=7 for AA deoxygenated cells with charybdotoxin, N=33 for SS oxygenated cells, N=19 for SS deoxygenated cells, N=8 for SS oxygenated cells with charybdotoxin, and N=5 for SS deoxygenated cells with charybdotoxin. Hyperpolarization magnitudes (mV) are obtained after the deduction of resting membrane potential (E_M) from the maximal hyperpolarization (V_{Max}) reached at each condition. (E) Resting membrane potentials, obtained at different conditions before the application of Δ^9 THC (80 μ M). N=12 for AA oxygenated cells, N=11 for AA deoxygenated cells, N=35 for SS oxygenated cells, and n=20 for SS deoxygenated cells. (F) Western blot showing the expression of TRPV2 and actin (as loading control) in healthy donors (AA1 and AA2), and SCA patients (SS1, SS2, and SS3) (top panel), and the quantitative comparison of band densities (ratio TRPV2/actin) on western blots from AA (N=2) and SS cells (N=3) (bottom panel). (G) Representative histograms of Fluo-4 intensity in high Ca^{2+} population of AA cells without Δ^9 THC (black trace), SS cells without Δ^9 THC (grey trace), AA cells with 80 μ M of Δ^9 THC (red trace), and SS cells with 80 μ M of Δ^9 THC (purple trace). (H) Quantitative analysis of AA and SS RBCs with high Ca^{2+} in response to 80 μ M of Δ^9 THC, N=8 for all conditions. All histograms represent means \pm SEM. Statistical difference was assessed using two-way ANOVA. (I) Elongation index (EI) in response to Δ^9 THC stimulation in patients' RBCs (paired t-test, *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001).

In the legend, “n” refers to the number of individual cells patched using Patch-Clamp technique and “N” refers to the number of independent patients/experiments.

-Supplementary Material-

Putative adverse effects of Δ^9 THC on sickle red blood cells

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Materials and methods

The study was based on a combination of different techniques: flow cytometry, MBE analysis, manual patch-clamp and ektacytometry.

Blood samples

All patients were followed at the Hospitals of Lyon (France). The study was conducted in accordance with the guidelines set by the Declaration of Helsinki and approved by the Regional Ethics Committees (L14-127, Université de Lyon). Blood from healthy donors was drawn into lithium-heparin vacutainer (BD Switzerland Sarl, Vaud, Switzerland) on site and SCA patients were drawn into EDTA vacutainer (BD Switzerland Sarl, Vaud, Switzerland) and shipped from Lyon to collaborating laboratories at room temperature.

Solutions and Chemicals

Flow cytometry experiments for intracellular calcium detection were performed on both patients and controls in Tyrode solution containing (in mM): 130 NaCl, 5 KCl, 5 Glucose, 10 HEPES, 1 MgCl₂ and 1.5 CaCl₂. pH was adjusted to 7.40 by NaOH.

The MBE method was conducted in Ringer solution with (in mM) 154 NaCl and 2 KCl. Carbonyl cyanide m-chlorophenyl hydrazine (CCCP) ionophore (Sigma-Aldrich, Saint Louis, MO, USA) was added at a final concentration of 20 μ M to increase the hydrogen permeability of membranes. Lysis' solution included Triton 1X and 3M of NaCl (Sigma Aldrich, St Louis, MO, USA).

Patch-clamp experiments were performed using the following solutions: pipette solution (in mM): 140 KCl, 2 MgCl₂, 10 Hepes; pH adjusted to 7.40 with KOH. Bath solution (in mM): 140 NaCl, 2 MgCl₂, 2 CaCl₂, 10 Glucose, 10 Hepes; pH adjusted to 7.40 with NaOH.

The ektacytometry method was performed with the EIon-ISO polyvinylpyrrolidone solutions (PVP, Mechatronics, The Netherlands).

To investigate the activation of the TRPV2 channel, we used the Δ^9 Tetrahydrocannabinol (Δ^9 THC) -ethanol solution (Sigma Aldrich, Saint Louis, MO, USA) or Δ^9 Tetrahydrocannabinol (Δ^9 THC) -methanol solution (LGC GmbH, Wiesel, Germany). The final concentration for each experiment is specified in the description of each method.

To investigate Gárdos channel interaction with TRPV2 activation, charybdotoxin (Alomone Labs, Jerusalem, Israel) was used as Gárdos channel inhibitor at a final concentration of 100 nM.

All salts were of analytical grade. Stock solutions of the compounds were dissolved according to the manufacturer's recommendations.

Flow Cytometry

Flow cytometry was used to assess the intracellular Ca²⁺ levels after the incubation of RBCs with Δ^9 THC. 25 μ L of blood were washed three times in the Tyrode buffer. RBCs were loaded with Fluo-4 AM (Molecular Probes, Eugene, OR, USA) at a concentration of 5 μ M for 1 h at 37°C with 400 rpm shaking. After washing three times in the Tyrode buffer, 2.5 μ L of Fluo-4 loaded RBCs were added to a 500 μ L designed Tyrode buffer in 5 mL round bottom polystyrene test tubes (Corning Inc., Corning, NY, USA). Flow cytometry measurements were performed on a BD FACSCanto™ II (BD Biosciences, Erembodegem, Belgium) or LSRFortessa (Becton Dickinson, San Jose, CA, USA).

To optimize the Δ^9 THC concentration for flow cytometry analysis and to investigate the kinetic properties of TRPV2 channel, a dose-response curve was realized on RBCs from 4 healthy donors using different final concentrations of Δ^9 THC (300 nM, 100 nM, 1 μ M, 3 μ M, 10 μ M, 30 μ M, 60 μ M, 90 μ M, 120 μ M). For each sample, data were recorded for 1 min directly after starting Δ^9 THC incubation.

For probing the effect of the selected Δ^9 THC concentrations (30 μ M and 80 μ M) on healthy and sickle RBCs, cell preparation was the same as above. For the Δ^9 THC treated group, data were recorded for 10 minutes directly after Δ^9 THC application. For control conditions in the Tyrode solution, 30000 cells were measured.

The results were assessed using Kaluza and Flow Jo (Becton Dickinson, San Jose, CA, USA).

Membrane Potential Measurements- Macey, Bennekou, and Egee method (MBE method)

As first described by Macey *et al.* (Macey RI, *Biomembr*, 1978), the use of CCCP ionophore permits to get the exact value of the membrane potential (V_M , in millivolts (mV)) by a measurement of the extracellular pH. This can be estimated using the following equation:

$$V_M = -61.5(pH_{in} - pH_{out}) \text{ at } 37^\circ\text{C}.$$

The blood was centrifuged at 2,500 g to discard the plasma and the buffy coat, then washed three times with Ringer solution. RBCs were centrifuged at 13,000 g for 2 minutes in order to reach 99.99% of hematocrit value (packed RBCs). Washed cells were conserved on ice until used for experiments.

100 μ L of packed RBCs were loaded into a beaker containing Ringer solution, pre-treated with 20 μ M of CCCP ionophore and tested with Δ^9 THC at two different concentrations: 30 μ M and 80 μ M for 230 s. To end the experiments, a lysis solution was added. To test Gárdos channel contribution, charybdotoxin was added at 200 s. The deoxygenated conditions were performed in deoxygenated Ringer solution, perfused with Nitrogen (N_2) gas before and during the experiment (oxygen concentration of 0.8 mg/L).

Recordings were made using the Logger Lite (Vernier; Millikan Way, Beaverton, OR 97005 France) program at an acquisition rate of 1 point/s.

Data were analyzed using Excel (Microsoft; Redmond- Washington, USA) and MATLAB R2022a.

Patch-Clamp

For the patch-clamp technique, 2 μL of whole blood were diluted in 500 μL of bath solution. Characterization of TRPV2 currents was performed in the whole-cell patch configuration. 3-5 G Ω seals were obtained by suction applied for less than 20 s. Patch pipettes (tip resistance ranging between 10 and 14 M Ω) were prepared from borosilicate glass capillaries (Clark Electromedical Instruments, England GC150F-10), pulled and polished on a horizontal programmable puller (DMZ, Werner Zeitz Augsburg, Germany). TRPV2 currents were elicited at room temperature by a 500 ms ramp voltage protocol from -100 to 100 mV, at a holding potential of 0 mV. After recording a stable baseline current in external solution (ES), cells were exposed to 30 μM $\Delta^9\text{THC}$ for 3 minutes, followed by application of 100 nM charybdotoxin. Current amplitudes and slope values (pA) were measured between 80 mV and 100 mV of the voltage ramp before and after compound addition for both patients and controls. If the current amplitude elicited upon $\Delta^9\text{THC}$ addition ($I_{\Delta^9\text{THC}}$) exceeded 3σ of the current amplitude at baseline conditions a cell was considered as $\Delta^9\text{THC}$ responder: $I_{\Delta^9\text{THC}} > I_{\text{ES}} + 3\sigma I_{\text{ES}}$. Traces were analyzed by Clampfit 10.7 (Molecular Devices LLC).

Ektacytometry of RBCs

Deformability of RBCs from patients and controls, with and without $\Delta^9\text{THC}$, was evaluated using the Deformability LoRRca protocol. The ektacytometry measurement is performed at 37°C in a Couette system, which consists of two separate concentric cylinders with a narrow gap between each other. The blood sample is placed in the gap between the two cylinders to be analyzed. During the measurement, the inner cylinder (called bob) remains static, while the external one (cup) moves in rotation creating a shear stress in a range of 0.3 to 30 Pa, which allows the deformation of RBCs. For the deformability measurement, 10 μL of RBCs were resuspended in 1 mL of PVP with 30 μM or 80 μM final concentration of $\Delta^9\text{THC}$ for 5 minutes. The PVP has a greater viscosity than the intracellular viscosity of RBCs, for this reason the RBCs can deform in the direction of the flow with an elliptical shape. A 670 nm red laser passes through the suspension and projects the resulting diffraction pattern onto a screen, and it is analyzed using LoRRca software provided by the manufacturer. From the diffraction spectrum of each blood sample, the software calculates the Elongation Index (EI) at each shear stress,

corresponding to the long and short axis of the ellipse: $EI = (\text{length} - \text{width}) / (\text{length} + \text{width})$.
Data obtained at 30 Pa are reported.

Data analysis

GraphPad Prism version 9 (GraphPad Software, San Diego, California USA) was used for the statistical analysis and presentation of the data. If data followed a Gaussian distribution (Shapiro-Wilk test), paired t-test or ordinary one-way ANOVA with multiple comparisons were used to test for statistical significance. Data are presented as mean \pm standard error of mean (SEM). If data did not follow a Gaussian distribution, the Wilcoxon test was used for comparisons between two datasets and the Kruskal Wallis test was used for multiple comparisons within the same dataset. $p < 0.05$ was deemed as significant.

8. Discussion

Sickle cell disease remains the most common genetic disease in the world, and although pathophysiological mechanisms are better known, there is still some unresolved issues regarding the modulators of the clinical expression.

The work of this thesis has led to the questioning of the influence of several factors on the pathophysiology of SCD, focusing on old (G6PD deficiency) and new (mitochondria) identified RBC oxidative stress sources and on the role of new identified non-selective cationic ion channels.

We observed that the coinheritance of SCA and G6PD deficiency resulted in greater cellular levels of oxidative stress, compared to those from patients with SCA only, but no difference in the other RBC physiological/rheological markers was observed. The reason why no other difference in other markers was reported could be due to some limitations of our present study. In our study, the results are based on a single cross-sectional analysis, rather than on repeated determinations. Furthermore, the results observed could be due to the small size of the group having the coinheritance of G6PD deficiency. Another reason could be that no molecular genetic investigation was performed to classify the G6PD alleles that could contribute to the different G6PD deficient phenotype. Different gene mutation could cause different levels of enzyme deficiency, with different classes assigned to various degree of deficiency and disease manifestation³³². The classification of the degree of deficiency could be useful because it has been observed that with complete G6PD deficiency, erythroid

precursors are unable to fight against high oxidative stress and they die by apoptosis, leading to a lower production of RBCs³³³. It could be possible that by performing an enzyme-deficiency degree discrimination, we might have seen differences in terms of hemolytic rates or in decreased hemoglobin concentration, due to a decreased production of RBCs. Furthermore, 100% of patients with G6PD deficiency were treated with HU, against the 77% of patients without G6PD deficit. HU significantly increases HbF production, improving RBCs volume and deformability and ameliorating the clinical status of the patients. This could be another explanation for the absence of differences between the two groups analyzed in this study. The effect of the coinheritance of G6PD and SCD is still debated in the literature, but our findings are in agreement with previous studies^{334,335}. However, even though our results do not associate the two diseases with worsened cellular features or clinical manifestations, it is essential that patients must know their status as carriers of both diseases to avoid consuming medications with pro-oxidant properties or substances that could worsen their clinical condition.

Even though G6PD deficiency seemed to have a limited impact on the physiological/rheological properties of RBCs from SCA patients, our results support an important role of the retention of mitochondria into mature sickle RBCs on cellular oxidative stress, RBC sickling and fragility. The abnormal presence of mitochondria in sickle RBCs is a recent discovery, and their functionality and contribution to oxidative stress are still debated in the literature. Indeed, the results of Martino et al.⁸⁷ reported a lack of association between the presence of mitochondria and oxidative

stress, prompting the authors to conclude that these residual mitochondria within sickle RBCs are not functional anymore. However, our results showed for the time that these mitochondria are still functional and contributes to ATP production and ROS generation. Whether it is the case also in patients with the HbSC genotype is unknown as we did not explore in detail the functionality of mitochondria in this SCD genotype. Our results also showed for the first time the association between the presence of mitochondria, lower deformability, and an increased propensity of RBCs to sickle at higher oxygen pressures. These findings could be of particular interest because sickle RBC deformability is a key parameter of blood flow into the microcirculation¹²⁰. In healthy individuals, RBCs are extremely deformable, capable of flowing through microcapillaries with a smaller diameter than their own, and any factors that may decrease their deformability could increase the risk of vaso-occlusions or organ damage in SCD.

Substantial progresses have also been made in elucidating the kinetics of HbS polymerization and RBCs sickling in hypoxic conditions, which have provided new strategies for developing anti-sickling drugs³³⁶. It is known that the repetition of the sickling process under oxygenation and deoxygenation leads to the formation of irreversible sickle RBCs, which may participate to vaso-occlusions, and any factor that promote sickling represents a risk. Our results suggest that the presence of still active residual mitochondria into mature sickle RBCs could increase the propensity of RBCs to sickle under deoxygenation. Active mitochondria use oxygen for oxidative phosphorylation, which could deprive hemoglobin S from oxygen, hence increasing

the risk for polymerization and RBC sickling. The repetition of sickling and unsickling cycles in the presence of functional mitochondria could further increase cellular damages and increase RBC fragility, which could explain the increased hemolytic markers reported in patients with the highest percentages of mature sickle RBCs retaining mitochondria. These findings are in agreement with those of Martino et al.⁸⁷ who also reported association between total bilirubin levels, reticulocyte counts and the presence of mitochondria.

Intravascular hemolysis represents a mechanism responsible for vascular disease³³¹ and many studies have confirmed the association of hemolysis markers with certain clinical complications of SCD³³⁷. The lack of association between mitochondria retention and clinical manifestations belonging to the hemolytic sub phenotype, such as priapism, glomerulopathy, stroke, leg ulcers or pulmonary hypertension, remains an interesting point that require further studies in larger cohorts.

We demonstrated that the presence of mitochondria was also associated with increased PS exposure in RBCs from HbSS and HbSC patients, proposing mitochondria as a new factor which could promote the premature senescence of sickle RBCs. Premature senescence of RBCs is a feature of SCD^{28, 338} characterized by increased percentages of RBCs exposing PS and high levels of intracellular ROS. Our study could suggest the contribution of mitochondria in the accumulation of intracellular ROS that, in turn may promote PS exposure on the outer leaflet of the red cell membrane, as ROS may directly activate scramblase³³⁹. Increased PS exposure is known to

promote RBCs adhesion to the vascular wall, contributing to vaso-occlusions²¹⁵. Another possible explanation for our results could be that the increased hemolysis which characterizes SCA patients could lead to an ineffective erythropoiesis to compensate for the massive loss of RBCs in the circulation, leading to the release of premature RBCs still retaining mitochondria in the blood circulation.

The abnormal retention of functional mitochondria is not the only factor that could compromise the rheological properties or promote the senescence of sickle RBCs. We also demonstrated a contribution of Piezo1 and TRPV2 channels on increased Ca^{2+} influx within sickle RBCs, RBC senescence and RBC sickling. Increased intracellular Ca^{2+} levels and different degrees of membrane hyperpolarization were observed after the stimulation of Piezo1 and TRPV2 channels, suggesting some similarities but also some differences between the two cationic ion channels. As demonstrated in our study, in experimental conditions the activation of Piezo1 is driven by Yoda1, a small molecule which acts as a molecular wedge facilitating force-induced conformational changes on the membrane. This mechanism of action suggests that Yoda1 is not totally specific for Piezo1. TRPV2 channel is also a mechano-sensitive protein³⁴⁰, rising immediate questioning on the putative simultaneous activation of the two cationic ion channels in sickle RBCs, and the consequent effects on RBC rheology and senescence. Indeed, by observing the membrane potential changes after the use of Yoda1, we noticed higher hyperpolarization levels, compared to those reported after the specific stimulation of TRPV2 by THC. Thus, one could hypothesize that the mechanical distortion of sickle RBCs membrane induced by Yoda1 has activated both

Piezo1 and TRPV2 channels, resulting in RBC rheology alterations and increased sickling propensity. Our studies also demonstrated that membrane hyperpolarization caused using Yoda1 or THC was far greater during hypoxia compared to normoxia, which support the fact that there would not only have one protein responsible for Psickle and that several cationic ion channels could be involved.

Furthermore, TRPV2 channel can be activated by THC or CBD derivates, opening questions about the use of CBD or THC derivates for the treatments of pain crises in SCD patients.

9. Perspectives

The work carried out in this thesis can be pursued in different directions. First, some questions regarding the functionality of the residual mitochondria within HbSC sickle RBCs remains open. Then, because the persistence of mitochondria into mature sickle RBCs is accompanied by RBC senescence and increased oxidative stress, we suspect that mitochondria retention into sickle RBCs could result in greater membrane blebbing and microparticles production. We are planning to discriminate sickle RBCs positive or negative for mitochondria and to evaluate the mitochondrial contribution to the production of microparticles. To do that, we could use a cell-sorting flow cytometer to separate sickle RBCs subpopulation retaining or not mitochondria. Doing a triple staining and setting a gating strategy in the cell-sorting flow cytometer, we could discriminate sickle RBCs retaining or not mitochondria (CD71- CD235a+ mitochondria+ RBCs and CD71- CD235a+ mitochondria- RBCs). Then, we could test the microparticles production challenging the two subpopulations of sickle RBCs with Antimycin A, the molecule we already used in the respirometry test, which inhibits the mitochondrial complex III and causes the increase of intracellular ROS production. After the incubation, we could wash and isolate the microparticles from the two subpopulations by ultracentrifugation. In this way, we could obtain the microparticles produced after the induction of mitochondrial ROS production, and we could analyze and quantify the microparticles by flow cytometry. Furthermore, we could investigate if mitochondria are also present into microparticles produced by RBCs, an interesting point which has never been investigated. However, the presence of mitochondria in

MPs from other types of cells has been reported: indeed, it has been observed that astrocytes secrete MPs containing mitochondria which are captured by stroke-affected neurons³⁴¹, suggesting that some types of human cells produce microparticles containing mitochondria.

Microparticles are an important hallmark of SCD, they participate to the pathophysiology of the disease by stimulating endothelial dysfunction and inflammation, and for this reason it could be important to evaluate new factors which impact the microparticles production from sickle RBCs retaining mitochondria. Isolating microparticles from mitochondria-positive and mitochondria-negative sickle RBCs, we could test their role on the activation of endothelial cells and compare the results obtained using the two different microparticles populations.

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