RED BLOOD CELL STRESS: A CHALLENGE TO HOMEOSTASIS

JOAMES KAUFFIMANN FREITAS LEAL

INVITATION

for attending the public defense of the doctoral thesis:

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Red blood cell stress:
a challenge to homeostasis

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Red blood cell stress: a challenge to homeostasis

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Red blood cell stress: a challenge to homeostasis

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Chapter 1

Introduction and outline of the thesis
From reticulocyte maturation to cytoskeleton dissociation, protein denaturation and appearance of new antigens, IgG binding, PS exposure, and vesiculation.
REDBLOODCELLOMEOSTASIS

Cellular stress is the wide range of physical and chemical insults that lead to molecular cellular alterations in response to environmental stressors, such as changes in temperature, reactive molecules, and mechanical shear. Most of the reactions to stress are intended to maintain cell structure and function and are dependent on stress severity and length\(^1\). Homeostasis is defined as the maintenance, by adaptation, of a reasonably stable internal setting as a consequence of a mutable external setting\(^3\). Physiological homeostasis of the red blood cell (RBC) compartment is maintained by a finely tuned balance between the generation of young, differentiated cells and removal of senescent, damaged cells\(^4\). At a concentration of 5 million RBCs per microliter of blood, red blood cells are the most abundant cell type in the circulation of the human body. In order to maintain RBC numbers, the bone marrow produces two million RBCs per second\(^5\). At this level of production, intrinsic and extrinsic factors controlling erythropoiesis must be carefully coordinated\(^6,7\).

Once in the circulation, the interactions between proteins in and associated with the plasma membrane play a central role in RBC homeostasis\(^8\). A molecular picture of the pathways that are involved in the process of RBC production and removal is gradually emerging: oxidative damage-induced, high-affinity binding of hemoglobin to band 3, activation of Ca\(^{2+}\)-permeable channels, phosphorylation-controlled impairment of metabolism and structure, degradation of band 3 and/or aggregation of band 3 fragments, binding of IgG, phosphatidylserine (PS) exposure, increased affinity for complement, and formation of microvesicles\(^9-13\). Some of these actors are illustrated in Figure 1.1.

It is becoming clear that the RBC contains a functional complex of regulatory systems that trigger interaction with and/or activation of the immune system after stress such as a change in osmotic conditions, and oxidative, mechanical or metabolic stress. Molecular information on the initial triggers of and cross-talk between the various pathways is rapidly increasing, and it is clear that senescent RBCs become more susceptible to stress, and that the modulation of the various pathways becomes progressively lost during aging. Similar changes may occur in patients with various RBC-centered or RBC-affecting diseases or exposed to various clinical conditions\(^5,9,10,14-17\).
Erythropoiesis

Erythropoiesis generates new RBCs at a constant rate. Erythropoiesis starts in the bone marrow with the multipotential hematopoietic stem cells, which can differentiate into progenitors of the common myeloid lineage, megakaryocyte/RBC progenitors, and finally into the burst-forming erythroid units (BFU-e)\(^{18,19}\).

This process is controlled by erythropoietin, produced in the kidney and liver, which induces proliferation of BFU-e and their differentiation into colony-forming unit-erythroid cells (CFU-e)\(^{18,20}\). In this differentiation phase, the erythroblasts mature in erythroblastic islands that are formed around a central nurse macrophage, in a process that involves multiple cell divisions, production of hemoglobin, removal of RNA, ribosomes, nucleus, and other organelles, and cell shrinkage, resulting in pyrenocytes and reticulocytes\(^{18-22}\). This maturation involves major membrane reorganization: the proteins associated with cellular adhesion such as Emp1 and β1 integrins are sorted toward the nucleus, which is expunged by the nurse macrophages as a pyrenocyte. The cytoskeletal proteins and integral membrane proteins essential for RBC shape and deformability such as ankyrin, spectrin, and the glycophorins are rearranged to form an enucleated cell containing high concentrations of hemoglobin\(^{21-24}\).

The reticulocytes exit into the bloodstream, where the expression of the transferrin receptor (CD71) is quickly downregulated through exosome release\(^{25-27}\), and the residual RNA content is removed by ribonucleases and subsequent exocytosis\(^{28}\) (Figure 1.1). Furthermore, final maturation is associated with re-organization of the cytoskeleton and membrane, resulting in a discoid shape and in the gain of the typical RBC deformability\(^{21,29-31}\).

RBC in the circulation – a lifetime of physiological stress

During its 120-day lifespan, the RBC travels approximately 120 miles, squeezing 160,000 times through capillaries of a third of its diameter\(^{5,32,33}\), and is always challenged by stressors that impair the structure of its cytoskeleton, its redox balance, and its membrane organization. Its specialized oxygen transport function combined with a lack of organelles have led to the development of exceptional means of survival\(^{10,12}\).
Red blood cell stress: a challenge to homeostasis

Red blood cell structure

RBCs are extremely elastic, stronger than steel in terms of structural resistance, and can deform with linear extensions of up to approximately 250%\textsuperscript{34,35}. Interestingly, however, an increase in surface area of only 3% to 4% results in cell lysis\textsuperscript{35}. All these abilities that contribute to RBC homeostasis result from its well-coordinated cytoskeletal network that enables the cell to experience large reversible deformations while maintaining structural stability\textsuperscript{34,35}.

The RBC membrane-skeletal structure is a hexagonal lattice, consisting of flexible alpha and beta-spectrin heterodimers that are linked to the membrane in two protein-complexes: the 4.1R or junctional complex and the band 3 or ankyrin complex\textsuperscript{36,37}. Each hexagon constitutes of six triangular elements. Every triangle accommodates three junctional complexes, one at each vertex, three band 3 complexes, one on each side, and contains four non-cytoskeleton-associated band 3 dimers\textsuperscript{38}.

Rearrangement of the cytoskeleton enables high deformability, as long as deformation occurs with a change in geometric shape at a constant surface area\textsuperscript{37,39–42}. Any further extension results in an increase of surface area and the breaking of junction points, resulting in vesiculation\textsuperscript{39,42} (Figure 1.1).

An abnormal increase in RBC rigidity has been associated with changes in the viscosity of the intracellular fluid, a decrease in the surface area-volume ratio, and in the membrane cytoskeleton and components. These processes all occur during physiological RBC aging\textsuperscript{43,44}. Disruptions that lead to weakening or breakage of the binding of ankyrin to band 3 affect RBC shape and decrease its stability\textsuperscript{38,45}. Also, the decrease in compression forces on the attached fluid membrane resulting from an unstable network may induce bending and eventual vesiculation. These conditions may occur during RBC aging in vivo and in in vitro and during ATP depletion or increased Ca\textsuperscript{2+} concentrations in in vitro\textsuperscript{41,46}.

Red blood cell redox balance

RBCs are well prepared to maintain redox balance with powerful antioxidant systems that are vital to keep hemoglobin in a reduced oxygen binding state and...
to limit oxidative damage by the superoxide anion, hydrogen peroxide, singlet oxygen and nitric oxide (NO)\(^47,48\).

Since the de novo synthesis of proteins and lipids is absent in RBCs, survival and metabolism rely entirely on the existing pool of biomolecules, and in the means to reverse damage or remove irreversible damaged and possible harmful components\(^35,41,49,50\). RBC aging is intimately linked to a gradual increase in irreversibly oxidized and denatured proteins, and a concomitant decrease in antioxidant activity, i.e. the concentration of reduced glutathione (GSH), ascorbate, vitamin E, NADH, and NADPH\(^48,51-54\). This leads to several disturbances, such as unbalanced calcium homeostasis\(^55-57\), formation of neoantigens\(^4,58\), diminished deformability\(^17,59-61\), and the appearance of phosphatidylserine exposure (PS) in the outer layer of the lipid bilayer\(^8,13,62\).

Oxidation of band 3 promotes phosphorylation, and dissociation of band 3 from the spectrin cytoskeleton\(^11,63,64\), resulting in enhanced mobility and generation of band 3 clusters with oxidized and denatured hemoglobin. These processes contribute to the appearance of a neoantigen that binds autologous immunoglobulin (IgG) and complement opsonins\(^13,59,65\). The oxidation of hemoglobin results in the formation of methemoglobin\(^65,66\), formation of Heinz bodies\(^67\) and peroxidation of membrane lipids\(^67,68\).

### Band 3

The anion exchange protein band 3 encompass 25% of the total membrane protein content, and with 2-3 million molecules per cell is the most abundant membrane protein of the RBC\(^69,70\). Deficits in band 3 and its interacting proteins are associated with disorders such as hereditary spherocytosis and hereditary elliptocytosis\(^17,45,71\).

Band 3 is a multifunctional protein: (1) transport of CO\(_2\) across the body, as it facilitates bicarbonate transport across the RBC membrane in exchange for chloride ions\(^72,73\); (2) binding site for glycolysis enzymes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), thereby involvement in regulation of ATP production\(^74\); (3) maintenance of cell shape and deformability, through its interaction with ankyrin, adducin, protein 4.1, and protein 4.2\(^73,75\).
Band 3 is susceptible to age-related damage, and oxidation-triggered phosphorylation, together with methemoglobin binding, resulting in the formation of high-molecular-mass band 3 clusters with neo-antigen activity. Moreover, phosphorylation of tyrosine residues on band 3 results in reduced interaction with ankyrin, and the concomitant weakening of the band 3-cytoskeleton bonds leads to an increase in lateral mobility followed by vesiculation (Figure 1.1).

**GPI-linked proteins**

In the outer layer of the RBC membrane, glycosylphosphatidylinositol (GPIs) anchor various molecules by covalent linkage. One of these proteins is acetylcholinesterase (AChE). Acetylcholine may play a significant role in hemorheological (vasodilatation) and oxygen-carrying properties, and nitric oxide metabolism and transport by RBCs. A decrease in AChE activity is seen as an enzymatic marker of RBC age. Reasons for this decrease may be membrane release by vesiculation processes and/or loss of enzyme function. Also, band 3 phosphorylation status has an impact on AChE activation, as band 3 protein dephosphorylation may inhibit AChE activity.

Other important RBC GPI-linked proteins are Decay Accelerating Factor (DAF, CD55) and Membrane Inhibitor of Reactive Lysis (MIRL, CD59). DAF promotes the rapid dissociation of C3 convertases of all three complement activation pathways inhibiting, then, C3b formation. MIRL protects cells from complement-mediated lysis through the binding of C9, thereby inhibiting its incorporation into the C5b-9 complex, which prevents the terminal polymerization of the Membrane Attack Complex (MAC) in the plasma membrane. Thereby, DAF and MIRL provide a protective barrier against complement activation and deposition.

MIRL and DAF have been widely studied because of their association with Paroxysmal Nocturnal Hemoglobinuria (PNH). PNH is a rare hematological disorder in which hematopoietic stem cells have a deficiency in MIRL and DAF, due to a mutation in the PIG-A gene that is responsible for GPI-linkage of these proteins. RBCs of PNH patients are very sensitive to complement activation, intravascular hemolysis and thrombosis.
Complement activation may play a role in RBC homeostasis, as aging has been suggested to be associated with increased deposition of C3b (Figure 1.1)\textsuperscript{33,93}. Formation of the DAF-C3b-GPA-band 3 complex has been speculated to induce increased coupling between the membrane skeleton and the lipid bilayer, as well as increased membrane viscosity, thereby enhancing macrophage recognition of the less deformable, senescent RBC\textsuperscript{94,95}. Furthermore, a subclass of IgG, with specificity for clustered band 3, is able to assemble an alternative complement pathway convertase that amplifies C3b deposition in \textit{in vitro}, thereby linking RBC phagocytosis to complement activation what might likely occur during ageing \textit{in vivo}\textsuperscript{13}.

**Lipids and phospholipid transport proteins**

The membrane lipid organization and composition are likely to play a role in RBC homeostasis, as suggested by the association of altered RBC morphology and lifespan in patients with hemoglobinopathies, liver disorders and chronic inflammation, in which the RBCs show increased exposure of phosphatidylserine\textsuperscript{96–98}.

The lipids in the RBC membrane consist of equal proportions of phospholipids and cholesterol\textsuperscript{35,99}. Phospholipid transport proteins maintain phosphatidylcholine (PC) and sphingomyelin (SM) in the outer layer and phosphatidylethanolamine and phosphatidylserine (PS) in the inner layer. ATP-dependent flippases transport PS and PE from the extracellular to the intracellular leaflet, whereas floppases do the opposite for PC and SM. Scramblases work both ways, depending on the concentration gradients, in a Ca\textsuperscript{2+}-dependent manner\textsuperscript{100,101}.

With a gradual increase in the intracellular calcium concentration, several mechanisms that have an impact on lipid organization are activated, i.e. scramblases, floppases, as well as calpain, calmodulin, the Gardos channel (potassium efflux), and protein kinase C, and flippases become less active\textsuperscript{102–104}. These reactions lead to the breakdown of lipid asymmetry, cell shrinkage, and are associated with vesiculation and RBC removal by macrophages (Figure 1.1)\textsuperscript{35,99}. In healthy individuals, only a few RBCs in the human circulation expose PS regardless
of their age, which may be interpreted as an indication that PS exposure leads to quick removal\textsuperscript{105,106}.

**RBC Removal**

One single RBC survives approximately 120 days in the circulation\textsuperscript{5,49}. In this period, the RBC volume (MCV) decreases, and the cellular hemoglobin concentration (MCHC) increases, mostly as a consequence of vesiculation\textsuperscript{50,59}.

The senescent RBCs undergo an efficient removal process: exposure of molecules that determine recognition by the immune system, i.e. senescent cell antigen on band 3, opsonization, phosphatidylserine in the outer leaflet of the membrane, and a decrease in CD47 and sialic acid\textsuperscript{9,10,13,58,77,107} all result in recognition and removal by macrophages of the reticuloendothelial system\textsuperscript{22}.

The continuous exposure to stress-induced perturbations of the RBC and its membrane during its lifespan has been related to the aging-associated decline of function, and ultimately recognition and removal of senescent cells by the immune system\textsuperscript{10,13}. In the spleen, besides RBC rheological impairments such as low deformability\textsuperscript{22,108}, the interaction of senescent RBC with macrophage receptors is pivotal on the maintenance of homeostasis. Fc receptors on the macrophages interact with senescence IgG; Stabilin-2 and Tim-4, TAM receptors interact with PS, and PS opsonins, CR1 and CR3 receptors interact with complement molecules\textsuperscript{22}. Altogether these receptors trigger phagocytosis and facilitate vesiculation\textsuperscript{109}.

Furthermore, RBCs contain surface proteins that may protect RBC from phagocytosis, i.e. the integrin-associated protein CD47\textsuperscript{110}. CD47 interaction with SIRP\textsubscript{α} in the macrophages acts as a “do not eat me-signal,” but age-related changes in CD47 concentration and/or conformation, that can be induced by oxidative stress, may lead to its low level and then trigger its clearance\textsuperscript{23,110,111}.

Also, the decrease in sialic acid with aging has a strong correlation with oxidation\textsuperscript{112,113}. Moreover, it has been suggested that the removal of sialic acid residues of the saccharide chains of glycoporphins results in recognition by antibodies, leading to the normal process of elimination of senescent cells by macrophages\textsuperscript{114,115} and/or allow adhesion molecules (Lu/BCAM) interaction with the
extracellular matrix allowing the capturing and removal of susceptible RBCs even under flow conditions\textsuperscript{116}.

The increase of “eat me signals,” and the decline in “do not eat me” signals, mark RBCs for removal. In general, the mechanical and biochemical conditions in the spleen, together with the presence of phagocytes, make this organ a quality control and repair system for RBC\textsuperscript{117,118}. Likewise, the liver has a significant role in the RBC removal by recognition of senescent cells by specialized macrophages\textsuperscript{18}.

**INDUCED STRESS AND HOMEOSTASIS**

Changes in the RBC environment that occur physiologically are frequently accompanied and/or aggravated by other stressors. Moreover, contact with non-natural surroundings may contribute to impaired homeostasis.

**Exercise**

Physical exercise induces acute oxidative stress, resulting in an increased level of oxidized biomolecules\textsuperscript{119,120}. In RBCs, exercise induces changes in viscosity, membrane fluidity, and rigidity, that all affect the deformability and thereby passage through capillaries\textsuperscript{121–128}. These changes may result from oxidative damage, that may affect especially the oldest cells in the population\textsuperscript{122}. Exercise-associated changes in shear stress, resulting from an increase in blood flow, may constitute other damaging factors\textsuperscript{129}.

**Stress in a non-physiological environment**

The most common non-physiological factors that have an impact on RBC homeostasis during medical interventions are mechanical trauma, non-physiological temperature, foreign surfaces, turbulence, cavitation, flow rate, and pulsatility as encountered in a clinical setting\textsuperscript{130}. RBC blood bank storage, transfusion, and circulatory assist devices are the most common, clinical sources of these stressors.
**Cardiopulmonary bypass environment**

Circulation through artificial organs and devices such as stents, artificial heart valves, ventricular assist devices, extracorporeal circulation in a heart-lung machine, and hemodialysis machines exposes RBCs to various stress conditions\textsuperscript{131}. The most significant factor in these non-physiological circulatory settings is a high level of shear stress and its associated mechanical damage, although other factors such as turbulence, cavitation, interaction/collision with foreign surfaces, and collisions between blood cells may also add to the resulting damage\textsuperscript{130}.

Extracorporeal circulation in cardiopulmonary bypass (CPB), in which blood is pumped through an oxygenator, is one of the most meaningful advances in medicine of the past century, but relatively little is known about its effects on RBC homeostasis\textsuperscript{132}. During extracorporeal circulation, the RBCs experience various forms of mechanical stress, induced by the diameter and surface coating of the tubing material, and the pump and oxygenator technology. These may lead to changes in deformability, membrane lipid composition, and organization that have a direct effect on tissue oxygenation and/or promote inflammation and coagulation\textsuperscript{132–135}.

**RBC concentrate storage environment**

Transfusion supports a wide variety of clinical treatments, and to be able to meet these broad needs RBC concentrates must be available safely, effectively and inexpensively\textsuperscript{136,137}. The possibility to store RBCs for reasonable time periods is convenient and lowers their cost. It also contributes to safety by allowing uniform donor screening standards, high sensitivity testing, and good component manufacturing procedures. However, the RBC concentrates are stored in an unnatural condition, i.e., at 4°C in a slightly hypertonic, acidic solution\textsuperscript{138–140} that may represent or develop into stressors over time in the blood bank.

Age-related changes of RBCs during cold storage are unavoidable, variable and nonlinear\textsuperscript{141,142}. Storage is associated with an increase in hemolysis and a leakage of potassium, an increase in volume and cell rigidity\textsuperscript{139,143}, changes in morphology\textsuperscript{144,145}, accumulation of microvesicles\textsuperscript{146–148}, depletion of ATP and 2,3-DPG\textsuperscript{141,144} aggregation and degradation of band 3\textsuperscript{10,149}, PS exposure\textsuperscript{150,151},
accumulation of hemoglobin, antioxidant and metabolic enzymes at the membrane⁸⁸–⁹⁰, degradation of proteins and decrease in spectrin and ankyrin¹⁰,¹⁵², and accumulation of oxidized proteins, in particular at the cytoskeleton¹⁵⁵. All those lesions contribute to transfusion side effects such as post-transfusion purpura, transfusion-associated circulatory overload, transfusion-associated dyspnea, transfusion-related acute lung injury, transfusion-associated graft-versus-host disease, and transfusion-transmitted infection¹⁵⁶.

Against this background, the chapters of this thesis present our new insights on RBC homeostasis under different stress conditions, to improve our understanding on adaptations RBCs undergo in vivo and in in vitro environments (Table 1.1).

<table>
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<th>Table 1.1: Following Chapters according to stress environment.</th>
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<tr>
<td><strong>In vivo</strong></td>
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<td>RBC Ageing (Chapter 2, 3)</td>
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<td>Disease (Chapters 2, 5)</td>
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<td>Exercise (Chapter 6)</td>
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OUTLINE OF THE THESIS

Red blood cell homeostasis in vivo is continuously challenged by stressors that cause biochemical and structural damage, which affect function and survival.

The general purpose of this thesis is to contribute to our understanding of the causes and consequences of the alterations that RBCs undergo in vivo in healthy individuals and in patients, and in in vitro during blood bank storage and during passage through a circulation-assisting heart-lung-machine.

Chapter 1 reviews the major actors on red blood cell homeostasis in physiological and non-physiological environments.

The generation of microvesicles is a response to stress that may prolong the lifespan of damaged, but still functional RBCs in physiological conditions in vivo. Chapter 2 aims to integrate the available data on microvesicle composition and production in vivo and in in vitro, their involvement in coagulation and inflammation processes, and their clinical importance.

Acetylcholinesterase (AChE), linked to the RBC membrane through a glycosylphosphatidylinositol (GPI) anchor, might not only be a marker for but may provide information on microvesicle generation as well. Chapter 3 describes our investigations on the structural and functional changes in AChE content during RBC aging in vivo, in comparison with aging in in vitro during storage in the blood bank. Our findings underline the disparities between aging in vivo and aging in in vitro, and illustrate the storage time-associated changes in microvesicle composition.

The studies described in Chapter 4 aimed to establish the effect of extracorporeal circulation (EC) on RBC physiology and homeostasis. In order to delineate the EC-induced changes, we measured various RBC homeostasis parameters in a stand-alone heart-lung machine and compared these with the RBCs from patients that were obtained during EC-assisted cardiac surgery. Our data show modifications in RBC biophysics through circulation in a heart-lung machine, both in in vitro and in vivo. Moreover, the degree and kinetics of these alterations are modified by the natural history of the RBCs, most conspicuously by the storage time span in the blood bank. Our findings may contribute to the establishment of a biologically rational platform for the improvement of extracorporeal circuit technology and surgical procedures in vivo.
Chapter 5 describes the results of an exploratory study on the role of GPI-linked proteins in RBC aging \textit{in vivo}. Using RBCs from patients with Paroxysmal Nocturnal Hemoglobinuria (PNH), we have employed our current knowledge of the molecular mechanisms involved in physiological RBC homeostasis to explore the effect of a total and partial absence of GPI-linked proteins on RBC structure, function, aging, and removal \textit{in vivo}. Our data indicate that the absence of GPI-linked proteins has no significant effects on RBC aging, but emphasize the importance of more detailed studies on the origin, composition, and biological activity of RBC-derived as well as platelet-derived microvesicles.

In Chapter 6 we describe the use of some hematological, functional and structural characteristics of RBC aging to test the hypothesis that exercise-associated stress \textit{in vivo} induces the fast removal of the oldest RBCs from the circulation. Our data show that this process occurs already during the first day of moderate-level exercise during the Four Day Marches of Nijmegen.

And in Chapter 7, the major findings and conclusions of this thesis are recapitulated, combined and discussed, especially regarding their implications for the future and limitations.
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Red Blood Cell Homeostasis: Mechanisms and Effects of Microvesicle Generation in Health and Disease

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Chapter 2

RBC homeostasis: vesiculation
Red blood cell stress: a challenge to homeostasis

ABSTRACT

Red blood cells (RBCs) generate microvesicles to remove damaged cell constituents such as oxidized hemoglobin and damaged membrane constituents, and thereby prolong their lifespan. Damage to hemoglobin, in combination with altered phosphorylation of membrane proteins such as band 3, lead to a weakening of the binding between the lipid bilayer and the cytoskeleton, and thereby to membrane budding and microparticle shedding. Microvesicle generation is disturbed in patients with RBC-centered diseases, such as sickle cell disease, glucose 6-phosphate dehydrogenase deficiency, spherocytosis or malaria. A disturbance of the membrane-cytoskeleton interaction is likely to be the main underlying mechanism, as is supported by data obtained from RBCs stored in blood bank conditions. A detailed proteomic, lipidomic and immunogenic comparison of microvesicles derived from different sources is essential in the identification of the processes that trigger vesicle generation. The contribution of RBC-derived microvesicles to inflammation, thrombosis and autoimmune reactions emphasizes the need for a better understanding of the mechanisms and consequences of microvesicle generation.
INTRODUCTION

Generation of microvesicles, i.e., extracellular vesicles that are shed from the plasma membrane\textsuperscript{157}, constitutes an integral part of red blood cell (RBC) homeostasis, and is responsible for the loss of 20\% of the hemoglobin and the cell membrane during physiological RBC aging \textit{in vivo}, and the accompanying decrease in cell volume and increase in cell density\textsuperscript{50,109}. The blood of healthy subjects contains approximately 1000 RBC-derived vesicles per microliter of plasma\textsuperscript{50,158–160}. The microvesicle hemoglobin composition suggests an enrichment of the irreversibly modified hemoglobins HbA1c and HbA1e2. Microvesicles contain various immunological recognition and removal signals\textsuperscript{50}, that are responsible for a rapid elimination – probably within minutes – from the circulation\textsuperscript{50,161}. Shedding of damaged cellular components by vesiculation prevents untimely removal of otherwise functional RBCs, as well as unwanted reactions of the hemostasis and immune systems. Thus, vesiculation couples general aging processes such as oxidation and glycation to organismal homeostasis. On the other hand, RBC-centered hemoglobinopathies such as sickle cell disease and thalassemia are accompanied by a substantial increase in microvesicle levels\textsuperscript{162,163}. Also, infection of RBC with the malaria parasite \textit{Plasmodium} induces vesicle formation. However, the underlying mechanism is likely to be strongly influenced by parasite-derived proteins, and therefore beyond the scope of this review (e.g., \textsuperscript{164}). Increased vesiculation is associated with systemic inflammation, which may be directly responsible for hemolysis and anemia\textsuperscript{98,165}. Thus, microvesicles are part of the complex of interactions between RBCs and the organism\textsuperscript{51,166}. In this review, we aim to integrate the newest data on microvesicle composition and production in various conditions, in order to obtain more insight into the basic mechanisms underlying microvesicle generation, and the involvement of RBC-derived microvesicles in pathophysiology.
maintenance of redox status such as catalase and peroxiredoxin-2, as well as large amounts of complement proteins and immunoglobulins\textsuperscript{162}.

The protein composition of microvesicles from patients with membranopathies, i.e., abnormal RBCs such as elliptocytes and stomatocytes due to genetic aberrations in membrane proteins, is likely to differ from those of control RBCs. Actual data are lacking, but this prediction can be deduced from the effects of splenectomy on the membrane protein composition of spectrin/ankyrin-deficient and band 3-deficient spherocytes\textsuperscript{171,172}. In RBCs from patients with thalassemia intermedia, hemoglobin damage may induce the formation of band 3 polymers, associated with increased phosphorylation that leads to a weakening of the band 3-ankyrin connection, resulting in microvesicle formation\textsuperscript{162}. These observations, together with the vesiculation-reducing effect of p72Syk kinase inhibitors, not only support a central role for the binding of modified hemoglobin, possibly especially to oxidized and/or proteolytically degraded band 3\textsuperscript{165,173} in the vesiculation process (Figure 2.1), but also show the involvement of phosphorylation networks in RBC homeostasis. The involvement of various signaling pathways in RBC vesiculation was supported by the relatively large numbers of signaling proteins in microvesicles obtained from the plasma of a healthy donor\textsuperscript{167}, and in a pharmacological screening \textit{in vitro}\textsuperscript{174}. 
Red blood cell stress: a challenge to homeostasis

Figure 2.1: Vesiculation in progress.

Structure of the RBC membrane during vesiculation, showing mechanisms involved in microvesicle shedding: breakdown of the cytoskeleton by calcium-dependent proteases; lipid bilayer rearrangement due to altered phospholipid transporter activities, which results in phosphatidylserine exposure; changes in band 3 configuration and distribution due to oxidation, binding of damaged hemoglobin, and phosphorylation, leading to loss of binding to the cytoskeleton at the ankyrin complex, recognition by IgG, and vesiculation. The order and interdependence of these processes are discussed in the text. PC, phosphatidylcholine; SM, sphingomyelin; PS, phosphatidylserine; PE, phosphatidylethanolamine.

THE INVOLVEMENT OF MICROVESICLES IN COAGULATION AND INFLAMMATION

Most RBC-derived microvesicles from healthy donors as well as from various patients expose phosphatidylserine, which promotes not only phagocytosis but also coagulation.

In vitro, thrombin generation through the intrinsic pathway has been shown to be induced by RBC-derived microvesicles derived from sickle cell patients, storage units, or after treatment with a calcium ionophore\textsuperscript{175–177}. In addition, correlations have been reported between the number of phosphatidylserine-exposing, RBC-
derived microvesicles, and thrombin generation in sickle cell patients\textsuperscript{175,178}. Microvesicles may also disturb anticoagulation reactions of the protein C system, possibly through binding of protein S (e.g.,\textsuperscript{179}). Increases in RBC-derived microvesicles in sickle cell disease and thalassemia patients is often accompanied by a decrease in deformability and hemolysis, which may as such constitute a risk factor for thrombosis.

Phagocytosis-triggered monocyte activation may induce proinflammatory and procoagulant endothelial cell responses\textsuperscript{180}. Thrombin may promote inflammation by activation of the complement system, e.g., by acting as C3 or C5 convertase\textsuperscript{181}. RBCs of patients with paroxysmal nocturnal hemoglobinuria (PNH) lack the GPI-anchored proteins CD55 and CD59 that protect against complement activation-associated hemolysis. GPI-anchored proteins may be involved in raft formation\textsuperscript{182}, and their absence may be directly responsible for the release of relatively high numbers of RBC-derived microvesicles with procoagulant activity in PNH patients \textit{in vitro}\textsuperscript{183–185}. In addition, microvesicles scavenge NO almost as fast as free hemoglobin and much faster than RBCs, which may impair vasodilation\textsuperscript{186}. This effect is already detectable with the number of microvesicles present in one transfusion unit\textsuperscript{187}.

\textbf{THE ROLE OF THE SPLEEN}

The spleen facilitates vesiculation, as apparent from the retention of microvesicles in RBCs in asplenic individuals. In these individuals, the normal aging-related decrease in total RBC hemoglobin is absent, due to an increase in HbA1c\textsuperscript{109}. In patients with spherocytosis, splenectomy increased RBC deformability \textit{in vitro}, probably by inhibiting spleen-mediated microvesicle shedding\textsuperscript{171}. The molecular mechanism underlying vesiculation in the spleen is unknown, but may involve a combination of biochemical and biophysical stress. Recent model simulations support the involvement of degraded hemoglobin in reducing the cytoskeleton/membrane connection, thereby promoting microvesicle shedding during splenic flow\textsuperscript{188}. Thus, the mechanical and biochemical circumstances in the spleen, together with the presence of specialized macrophages, may make the spleen a microvesicle-based quality control and repair system. This emphasizes the importance of establishing the functionality of the spleen, especially in the study of
diabetic control. Also, the notable paucity of data for microvesicles generated in vivo warrants a more detailed investigation on the fundamental and clinical relationship between splenectomy or functional asplenia, RBC-derived microvesicles and RBC homeostasis.

**MICROVESICLES IN VITRO**

Vesiculation also occurs during storage of RBCs in the blood bank. Storage microvesicles contain removal signals such as phosphatidylserine in the outer layer of their membrane and degraded as well as aggregated band 3 molecules, similar to microvesicles in the circulation. In blood bank microvesicles, the number of carbonyl groups is increased relative to RBC membranes, possibly due to the accumulation of oxidized membrane proteins band 3, actin and protein 4. Blood bank microvesicles are immunologically active, as they contain immunoglobulins and complement factors, derived from the plasma fraction of the storage fluid. Also, storage microvesicles are readily recognized by pathological autoantibodies from patients with autoimmune hemolytic anemia. These data indicate that the coupling of removal of damaged components from the RBC to their removal from the circulation is a general phenomenon for RBC-derived microvesicles. The enrichment of the GPI-anchored proteins acetylcholinesterase and CD55, as well as raft-associated forms of stomatin and the flotillins in storage microvesicles, indicates that lipid-related changes in membrane organization are involved in vesiculation during storage. The underlying mechanism has been proposed to be revolving around membrane budding and fission. This could be triggered by the loss of binding between cytoskeletal and membrane proteins, followed by large-scale separation of various lipid phases that may be formed by membrane protein-stabilized microdomains. The loss of interaction between the cytoskeleton and cell membrane may be triggered by oxidized hemoglobin, similar to what may happen in vivo. Indeed, accumulation of oxidized hemoglobin residues during storage is accompanied by their enrichment in microvesicles. This role of hemoglobin in microvesicle formation is supported by the observation that, in the early phase of storage, a significant amount of hemoglobin is associated with the lipid bilayer in
There is a shortage of detailed quantitative and qualitative information on the primary triggers driving microvesicle production in vitro. The available data, albeit mostly showing associations, support a role of phosphorylation and rearrangement of band 3. For example, inhibition of tyrosine dephosphorylation not only induces RBC shapes such as echinocytes, which indicates a loss of interaction between the cytoskeleton and the lipid bilayer but also stimulates microvesicle production in vitro\textsuperscript{8,11,162}. In the misshapen cells found in patients with neuroacanthocytosis, disturbed phosphorylation and altered cell morphology are accompanied by disturbed microvesicle generation\textsuperscript{195}. Phosphorylation of band 3 is associated with clustering and correlates with microvesicle formation during storage and in the RBCs of patients with thalassemia intermedia\textsuperscript{162,196}. Similar effects are observed upon treatment of RBCs with agents that induce aggregation of band 3\textsuperscript{8,11}. A well-known stimulus for microvesicle formation in vitro is an artificial increase in intracellular calcium concentration. However, the protein composition of calcium-induced microvesicles differ from storage or blood microvesicles, e.g., the content of membrane proteins, the presence of band 3 aggregates and breakdown products, and of raft-associated proteins\textsuperscript{190,193,197}. This indicates that alterations in intracellular calcium concentrations are not primary factors in microvesicle generation in vivo, nor in the blood bank.

**MECHANISMS OF VESICULATION: INVOLVEMENT OF LIPIDS**

The few data that are available indicate that disturbances of the organization of the lipid part of the cell membrane may play a role in the vesiculation process. The RBC membrane contains sphingomyelin/cholesterol-enriched as well as cholesterol-enriched domains that are associated with high-curvature areas. Since these domains become associated with budding membrane areas during storage at 4°C, they have been speculated to be specific sites of microvesicle generation\textsuperscript{99}. However, RBCs and microvesicles obtained during storage in blood bank conditions showed no significant differences in the main phospholipid classes\textsuperscript{146}. This included the lack of enrichment of the raft-associated lipids cholesterol and sphingomyelin. Thus, lipid-involving reorganizations in the RBC membrane may be instrumental in microvesicle generation, but they do not seem to result in significant
alterations in microvesicle lipid composition. Changes in membrane lipid organization, such as an increase in exposure of phosphatidylserine and/or phosphatidylethanolamine, may promote vesiculation during storage \(^{198,199}\). However, severe disruptions of the protein-protein interactions, that are associated with altered RBC morphology, may induce increased microvesicle generation, but are not always accompanied by increased phosphatidylserine exposure \(^8\) (Figure 2.1). In this context, it should be emphasized that not all RBC-derived microvesicles expose detectable amounts of phosphatidylserine \(^{50,200}\). Disruption of the lipid bilayer, e.g., by treating RBCs with sphingomyelinase, strongly catalyzed microvesicle generation \textit{in vitro}. This process was accompanied by the appearance of CD59 and stomatin clusters in the RBCs, supporting a role for lipid rearrangement in microvesicle formation. The sphingomyelinase-induced microvesicles were much more heterogeneous in phosphatidylserine exposure and glycophorin A content than the microvesicles generated by spontaneous vesiculation, indicating the involvement of different mechanisms \(^{165}\). Thus, changes in lipid organization may facilitate microvesicle formation, but may not constitute the primary mechanism in most physiological conditions.

**MECHANISMS OF VESICULATION: COMPARISON WITH EXOSOME FORMATION**

All reports on RBC-derived microvesicle composition, especially in combination with the aging-associated changes in the RBC membrane proteome, indicate the involvement of proteins that are involved in the release of exosomes as well \(^{157}\). This includes small GTPases, lipid raft-associated proteins such as acetylcholinesterase and flotillins, and annexins \(^{157,167,197}\). Although it is not clear how cytosolic components end up in exosomes, the mechanisms by which cytosolic molecules are recruited into RBC-derived microvesicles may be similar to those involved in exosome generation, as indicated by the presence of various chaperone proteins \(^{157,167}\). The molecular details of the mechanisms underlying microvesicle generation in other cell types are largely unknown. Incorporation of the available data on RBC-derived microvesicles into the catalog ‘Vesiclepedia’ \(^{201}\) may be a worthwhile first step toward further elucidation of the mechanism of microvesicle in RBCs, as well as in other cell types. A comparison of RBC microvesicle data with the
already available data on RBC exosomes that are shed by reticulocytes from human cord blood\textsuperscript{202} will facilitate the identification of the molecular mechanisms involved in various types of vesiculation \textit{in vivo}. Already, endocytosis and autophagy have been involved in the disappearance of CD71 and other membrane proteins during reticulocyte maturation \textit{in vitro}\textsuperscript{29}. Such an approach profits from the possibility that RBCs create to study exosome as well as microvesicle formation during differentiation and aging in the same cell, that has a relatively homogeneous and well-charted membrane system.

**FROM MECHANISM TO MARKER TO MEDICINE**

Red blood cells form microvesicles in response to a variety of physiological and pathological triggers. Although the inventory of the composition of microvesicles generated in different circumstances is far from complete, the available data indicate that they all are enriched in damaged RBC components, depending on the various stimuli (Figure 2.1). This suggests that an exhaustive study of RBC-derived microvesicles will offer insights into the molecular mechanisms of their generation \textit{in vivo}, and thereby into the physiological and pathological triggers. Also, RBC-derived microvesicles may constitute a model for the study of the biological, biophysical and clinical properties of microvesicles in general. This model will benefit from the comparison of the composition and characteristics of RBC-derived microvesicles with microvesicles generated by other cell types, and with exosomes. RBC-derived microvesicles are potentially sensitive and specific biomarkers for the clinical severity of RBC-centered diseases such as sickle cell disease, thalassemia or spherocytosis\textsuperscript{171,203,204}. Also, microvesicles may reveal the activity as well as the clinical consequences, such as anemia or thrombosis, of systemic processes, such as inflammation. In addition, RBC-derived microvesicles may be useful in the transfer of surface proteins, as has been shown in the ‘painting’ of RBCs of PNH patients with the complement-protecting proteins CD55 and CD59\textsuperscript{205}.

Where RBC-derived microvesicles may be actively involved in pathology, e.g., by their procoagulant, proinflammatory or autoimmune activity\textsuperscript{206}, pharmacological prevention of the formation of harmful microvesicles may become of clinical importance. The recent finding that inhibition of sphingomyelinase attenuated lung inflammation caused by infusion of stored RBC-derived microvesicles\textsuperscript{207}, supports
this notion. Thus, on one hand, microvesicle shedding may prevent the untimely removal of functional RBCs in physiological conditions. On the other hand, in pathological conditions, prevention of vesiculation following splenectomy may have beneficial effects, or prevent a pathological immune reaction, for example after massive or frequent RBC transfusion in compromised, transfusion-dependent patients.

**AUTHOR CONTRIBUTIONS**

GB conceived the topic and wrote the final version. GB, JL, and MA-H wrote parts of the manuscript.

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**CONFLICT OF INTEREST STATEMENT**

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.
RBC homeostasis: vesiculation
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Acetylcholinesterase provides new insights into red blood cell aging *in vivo* and *in vitro*

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Chapter 3

How RBC homeostasis is affected by blood bank storage
Background: During its 120 days sojourn in the circulation, the red blood cell (RBC) remodels its membrane. Acetylcholinesterase (AChE) is a glycosylphosphatidylinositol (GPI)-linked enzyme that may serve as a marker for membrane processes occurring this ageing-associated remodeling process.

Materials and methods: Expression and enzymatic activity of AChE were determined on RBCs of various ages, as obtained by separation based on volume and density (ageing in vivo), and on RBCs of various times of storage in blood bank conditions (ageing in vitro), as well as on RBC-derived vesicles.

Results: During ageing in vivo, the enzymatic activity of AChE decreases, but not the AChE protein concentration. In contrast, neither AChE activity nor concentration show a consistent, significant decrease during ageing in vitro. CD59, another GPI-linked protein that protects against complement-induced removal, also remains constant during storage. The cellular content of the integral membrane protein glycophorin A, however, decreases with storage time in the more dense RBC fractions. The latter are enriched in echinocytes and other misshapen cells during storage.

Discussion: Our findings suggest that, during RBC ageing, GPI-linked proteins and integral membrane proteins are differentially sorted. Also, the vesicles that are generated in vitro show a fast and extensive loss of AChE activity, but not of AChE expression. Thus, AChE characteristics may constitute sensitive biomarkers of RBC ageing in vivo, and a source of information on the structural and functional changes that GPI-linked proteins undergo during ageing in vivo and in vitro. This information may help to understand RBC homeostasis and the effects of transfusion, especially in immunologically compromised patients.
INTRODUCTION

During its life of 120 days in the circulation, functionality of the red blood cell (RBC) is maintained and cell removal is postponed by disposal of modified haemoglobin and damaged membrane components through vesiculation. When vesiculation capacity has reached its limits, the ongoing mechanical, osmotic and oxidative stress induces recognition and removal of the old RBC by the immune system. The same mechanisms that cause the decrease in deformability, adenosine triphosphate (ATP) production and maintenance of redox status, are also likely to be responsible for the generation of removal signals. This process revolves around band 3 and its partners.

Although considerable progress has been made in the characterization of RBCs of various ages by comparative proteomics and metabolomics, the molecular details of the ageing mechanism(s) remain elusive. This is mainly due to the practical and ethical limitations of manipulating RBCs in healthy volunteers. Insights obtained from investigating patients with RBC-centered pathologies are obscured by disease-specific and systemic effects. Thus, storage in blood bank conditions may be the most valid model for the elucidation of RBC ageing processes, with the highest translational value.

In all conditions, the study of RBC ageing is hampered by the absence of an easily determined, unambiguous, general age marker. Acetylcholinesterase (AChE), linked to the RBC membrane through a glycosylphosphatidylinositol (GPI) anchor, might be such a marker for RBC ageing in vivo. Determination of AChE characteristics may also yield information on the mechanism of ageing-associated vesicle formation. Microvesicles generated after an increase in intracellular calcium concentration or after mechanically-induced membrane deformation in vitro have been described to be enriched in AChE as well as other GPI-anchored proteins such as CD55 and CD59, with a concomitant depletion of these proteins in the cellular membrane. In addition, we have found that the membrane distribution of AChE in vesicles generated during storage in blood bank conditions changes with storage time. Therefore, we investigated the structural and functional changes that AChE undergoes during RBC ageing in vivo, and compared this with ageing in vitro during storage in blood bank conditions.
MATERIAL AND METHODS

Red blood cell sampling

Fresh RBCs were isolated from 5 mL whole EDTA-blood from healthy volunteers. Written informed consent was obtained from all donors. The study was performed following the guidelines of the local medical ethical committee and in accordance with the Declaration of Helsinki. Storage experiments were performed with RBCs from 5 leucodepleted red cell concentrates (3 blood group A RhD-positive donors, 1 blood group A RhD-negative donor, 1 blood group O RhD-positive donor), as described before\textsuperscript{151}. Samples of 10 mL were taken at 1, 7, 14, 21, 28 and 35 days after blood collection. RBCs were isolated from fresh blood and leukocytes and platelets were removed as described before\textsuperscript{151}. RBCs were extensively washed three times with Ringer (125 mM NaCl, 5 mM KCl, 1 mM MgSO\textsubscript{4}, 32 mM Hepes, 5 mM glucose, 2.5 mM CaCl\textsubscript{2}, pH 7.4) by repeated centrifugation for 15 min at 700 g.

Red blood cell fractionation

Red blood cells were fractionated according to cell volume followed by a fractionation according to cell density as described earlier\textsuperscript{220}, and fractions were combined to achieve 5 fractions ranging in cell age from young to middle-aged to old cells, as determined by their HbA1c content\textsuperscript{50}. Red blood cell fractionation according to density was performed using a discontinuous Percoll gradient consisting of six layers ranging from 0% Percoll (1.060 g/mL) to 80% Percoll (1.096 g/mL) and the resulting layers were combined into top, middle and bottom layer fractions, basically as described before\textsuperscript{151,220}.

Acetylcholinesterase activity assay

Acetylcholinesterase activity of intact RBCs at a concentration of 2×10\textsuperscript{6} cells/mL and RBC-derived vesicles was measured using a modified Ellman’s method under $V_{\text{max}}$ conditions, basically as described before\textsuperscript{216,219}. The AChE activity of vesicles was measured using the supernatant obtained after centrifugation for 30 min at 2,880 g in the presence of the butryrylcholinesterase inhibitor tetraisopropyl pyrophosphoramidate (0.1 μM, Sigma Life Sciences, Zwijndrecht, The Netherlands).
One arbitrary AChE enzyme unit was defined as an increase in absorbance at 412 nm of 0.005 per minute, determined from the slope of the initial part of the absorption vs the time curve that was obtained by linear regression analysis.

**Flow cytometry**

The presence of AChE on RBCs was examined by flow cytometry using goat anti-AChE ab34533 (biotin, 1:25; Molecular Probes, ABCAM, Uithoorn, The Netherlands), detected by a rabbit anti-goat labeled with Alexa 488 (1:200; Molecular Probes). RBCs were analysed with a flow cytometer (FACScan; Becton Dickinson, Franklin Lakes, NJ, USA) and its accompanying software (CELLQUEST, Becton Dickinson). The results are expressed as percentages of AChE-positive RBCs and/or their mean fluorescence intensity (MFI). Vesicles were isolated and analysed by flow cytometry, as described previously.

**Microscopy**

Cells were suspended in Ringer and RBC morphology was assessed by classifying the cells in normocytes, echinocytes, cells of indistinct morphology (misshapen cells), stomatocytes, macrocytes, spherocytes, ovalocytes and elliptocytes in random field pictures, using confocal laser microscopy, as described before. At least 100 cells were analysed per sample.
RESULTS

In order to investigate the structural and functional changes in acetylcholinesterase upon ageing in vivo and in vitro, we started by determining enzymatic activity. RBCs isolated from whole blood and separated according to cell age by a double separation method showed a decrease in activity with increasing cell age (Table 3.1). Similar differences were observed when cells were separated based on differences in cell density only (Table 3.1). This loss of activity could be due to a selective loss of AChE-containing membrane by vesiculation. Quantitation of AChE content by flow cytometry, however, showed no differences between the various cell fractions (Table 3.1). These data indicate that RBC ageing in vivo is not accompanied by a selective loss of AChE protein, but by a decrease in enzyme activity.

In contrast, during ageing in vitro, i.e. during storage as red cell concentrates in the blood bank, the mean AChE activity of RBCs did not undergo a statistically significant change with storage time (Figure 3.1A). However, there was considerable variation between the red cell concentrates (data not shown), probably due to biological, inter-individual variability in the response to the artificial storage conditions150,151. Measurement of the AChE activities of RBCs in density-separated fractions did not show a consistent correlation between enzyme activity and density, although there was a significant decrease in the most dense cell layer after 21 days of storage, which was not observed at later time points (Figure 3.1B). These observations are in stark contrast to the differences between the activity of Percoll-separated RBCs of fresh samples, i.e. before they underwent the red cell concentrate production procedure. Already on the first day, the AChE activity of the least dense RBCs was significantly reduced compared with the activity of fresh RBCs (Figure 3.1C). In contrast to the RBCs, pronounced changes were observed in RBC-derived vesicles. During storage, the vesicle concentration increases from a vesicle/RBC ratio of 1.0 at days 7 and 14 to a vesicle/RBC ratio of 1.5 at day 21, and a vesicle/RBC ratio of 4.3 on day 35, as has been shown before193. The AChE activity of these vesicles, however, showed a strong, steady decrease during storage (Figure 3.1D).
Table 3.1: Red blood cell acetylcholinesterase activity during aging in vivo.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Double separation</th>
<th>Density separation</th>
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<td></td>
<td>AChE activity ± SD</td>
<td>Layer</td>
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<tr>
<td>Young</td>
<td>100</td>
<td>Top 100</td>
</tr>
<tr>
<td>Middle-aged</td>
<td>83.70 ± 30.71</td>
<td>Middle 79.70 ± 16.01</td>
</tr>
<tr>
<td>Old</td>
<td>68.73* ± 18.34</td>
<td>Bottom 61.23* ± 8.53</td>
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Acetylcholinesterase (AChE) activity was measured for RBCs separated according to cell age using double separation, and on RBCs separated according to density using Percoll gradients (Materials and Methods). Enzyme activity, expressed as arbitrary units, and protein content, expressed as mean fluorescence intensity (MFI), were determined using a spectrophotometric assay and flow cytometry, respectively (see Material and Methods). *, p<0.05, as determined by ANOVA, Tukey’s Multiple Comparison Test, as compared with the young fraction or top layer. SD: standard deviation; N: number; RBC: red blood cell.
Figure 3.1: Red blood cell acetylcholinesterase activity during storage in blood bank conditions.
A, Acetylcholinesterase (AChE) activity of unseparated RBCs during storage; B, AChE activity of RBCs during storage after Percoll separation per layer; C, Comparison of AChE activity of Percoll-separated fresh RBCs and RBCs after one day of blood bank storage; D, AChE activity of RBC-derived vesicles expressed as arbitrary unit /total vesicle protein during storage RBCs and vesicles were isolated and their AChE activity was measured as described in Material and Methods. *p<0.05, **p<0.01, ***p<0.001. A, B, D, Tukey’s Multiple Comparison Test, ANOVA; C – Student t-test.
How RBC homeostasis is affected by blood bank storage

CD59, another GPI-linked protein showed no change in cell content with storage time, neither in the whole population nor in the various cell fractions (Figure 3.2A). Similar data were obtained with an anti-AChE antibody and comparable data have been obtained for ageing in vivo50. In addition to GPI-anchored proteins, we
included the integral membrane protein CD235a (glycophorin A) in our analyses. Even though there were no storage-associated changes in the total cell population, there was a decrease in glycophorin A content in the higher density RBCs of the middle and bottom Percoll layers starting after one week of storage (Figure 3.2B). These data indicate a selective disappearance of integral membrane proteins upon storage.

Ageing in vivo as well as ageing in vitro have been described to be accompanied by changes in cell shape\textsuperscript{213,220}. We performed a semi-quantitative analysis of RBC morphology during storage, both in unseparated samples and in the RBC fractions obtained after density separation. Our data confirm previous data, showing an overall decrease in the fraction of normocytes when storage time increases, with a concomitant increase in the numbers of echinocytes and otherwise misshapen cells (Figure 3.3). Morphological analysis of the various Percoll layers showed considerable variation, even within one red cell concentrate during storage (data not shown), but in general this change occurred in all layers (Figure 3.3A and B), and the most dense layers are enriched in echinocytes already after one day of storage (Figure 3.3A). We found no statistically significant correlations between cell morphology, AChE activity or AChE content.

![Figure 3.3: Red blood cell morphology during storage in blood bank conditions.](image)

RBCs were classified as normocytes, echinocytes, unclassified misshapen cells, or ‘others’ (ovalocytes, elliptocytes, stomatocytes).
How RBC homeostasis is affected by blood bank storage

DISCUSSION

Flow cytometry measurements of protein expression show no alterations in cellular AChE content with ageing in vivo (Table 3.1). Together with previous data on other GPI-linked proteins such as CD55 and CD59, as confirmed here, these findings suggest that there is no specific loss of GPI-linked proteins from the RBCs by vesiculation in physiological circumstances. Thus, previous findings suggesting selective sorting of GPI-linked proteins and microdomain-associated proteins during RBC vesiculation are likely to be related to the method that was employed to generate such vesicles in vitro. This conclusion is supported by proteomic data that show selective membrane and cytoskeletal protein sorting during vesicle formation, but also indicate that different mechanisms underlie vesicle generation in vivo and in vitro. In contrast, AChE enzyme activity clearly decreases with ageing in vivo. Such a decrease was already visible after Percoll separation only (Table 3.1). Since this density-based separation generates fractions that are only moderately enriched with young but not with older cells, this finding strongly suggests that the first and major loss of enzyme activity in vivo occurs relatively early in RBC life. Thus, AChE activity, but not AChE amount, constitutes a sensitive biomarker of RBC ageing in vivo. This apparent divergence between changes in expression and activity of one GPI-linked protein indicates that the absence of a clear ageing-associated decrease in CD55 and CD59 content, as has been reported previously, does not necessarily imply sustained protection against complement activation-associated opsonization during ageing. Thus, during ageing in vivo, CD59 may very well undergo conformational changes that decrease its capacity to shield the RBC from the complement system. This implication of our findings warrants further investigation of the role of complement in RBC removal in physiological conditions.

The conspicuous discrepancy between AChE protein content and enzymatic activity becomes even more pronounced in the vesicles that are produced with increasing storage time (Table 3.1 and Figure 3.1D). This apparent enrichment of inactivated AChE in vesicles confirms previous proteomic and immunological data showing a removal of damaged membrane components by vesiculation, both in vivo and in vitro. Thus, vesicles in red cell concentrates may constitute a rich and relatively pure source of damaged AChE, which may facilitate a molecular identification of the processes that cause the underlying structural alterations during
ageing in vivo and in vitro. Such an identification will help in understanding the unwanted side effects of transfusion, especially with regard to complement-mediated hemolysis in susceptible patients.

The data obtained by determination of AChE expression and activity during RBC storage in blood bank conditions emphasise the differences between ageing in vivo and ageing in vitro. In general, neither AChE expression nor enzymatic activity decreased significantly with storage time (Figure 3.1A). This result is in agreement with previous data showing no significant or only small alterations in AChE activity after prolonged storage times\textsuperscript{223}. Also, there were hardly any consistent differences between the RBCs of the different Percoll layers (Figure 3.1B). Many other observations show storage-associated changes in morphology (Figure 3.3\textsuperscript{153}), metabolism and membrane protein composition\textsuperscript{210,216}. A large inter-individual variability, together with an altered osmotic behaviour in response to the storage medium\textsuperscript{193}, are likely to obscure any ageing-related changes occurring in blood bank conditions. Data on phosphatidylserine exposure support these hypotheses, as does the absence of a decrease in cell volume during storage, in spite of the large increase in vesicles\textsuperscript{149,193,209,224}. These phenomena also hamper the interpretation of the flow cytometry data on CD59 and glycophorin A content with storage (Figure 3.2). A comparison of the various density fractions, however, suggests that during storage integral and lipid-linked membrane proteins undergo different fates when the RBCs change from discocytes to echinocytes (Figures 3.2 and 3.3). Such fates are likely to be dependent on selective removal by vesiculation.

The relatively large difference in AChE activity between fresh and 1-day old RBCs (Figure 3.1C) is likely to be caused by loss of AChE in vesicles that are generated during the mechanical stress that the RBCs undergo during the production of the red cell concentrates. The small, but significant increase in phosphatidylserine-exposing RBCs during blood bank processing and/or in the first week of storage supports this theory\textsuperscript{150}. 
How RBC homeostasis is affected by blood bank storage

CONCLUSIONS

The physiological role of RBC-bound AChE is not clear, but our findings may provide new tools for studying the putative role of acetylcholine on RBC physiology, and the impact of transfusion on acetylcholinesterase-mediated RBC homeostasis and survival, and on the circulation\textsuperscript{62,86,88,225}.

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AUTHORSHIP CONTRIBUTIONS

JKFL and GJB performed the experiments, JKFL, MJW A-H, RB and GJB designed the experiments and JKFL, MJW A-H, RB and GJB discussed the results and wrote the manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.
Red blood cell stress: a challenge to homeostasis

Submitted for publication as:
The impact of circulation in a heart-lung machine on function and homeostasis characteristics of red blood cells.

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Chapter 4

How RBC homeostasis is affected by extracorporeal circulation-assisted surgery
ABSTRACT

Extracorporeal circulation is accompanied by changes in red blood cell morphology and structural integrity that may contribute to the various side effects of heart-lung machine-assisted surgery. Our main objectives were to determine the effect of circulation of red blood cells in a stand-alone heart-lung machine on parameters of red blood cell aging, and to compare this with the effect of extracorporeal circulation-assisted cardiac surgery. Our results show that circulation in a heart-lung machine is accompanied by changes in red blood cell volume, an increase in osmotic fragility, changes in deformability and aggregation, and alterations in exposure of phosphatidylserine and in microvesicle generation. These changes in key characteristics of the red blood cell aging process increase the susceptibility of red blood cells to the various mechanical, osmotic and immunological stress conditions encountered during and after surgery in the patient’s circulation, thereby contributing to the surgery side effects. Thus, aging-related parameters in red blood cell structure and function provide a foundation for validation and improvement of extracorporeal circulation technology.
INTRODUCTION

Maintenance of oxygen supply by extracorporeal circulation (EC), for example during coronary bypass grafting surgery in a heart-lung machine, creates various non-physiological conditions that contribute to post-operative complications such as thrombosis, intravascular hemolysis, and inflammation. These complications affect perfusion and oxygen delivery, induce anemia, and may contribute to short-term as well as long-term defects in hemostasis, and to cognitive decline and other neurological problems. Changes in red blood cell (RBC) function and homeostasis are likely to contribute to these symptoms. During EC, the RBCs experience various forms of mechanical stress, induced by the diameter and surface coating of the tubing material, the pump and the oxygenator technology that may lead to changes in deformability, membrane lipid composition and organization that have a direct effect on tissue oxygenation and/or promote inflammation and coagulation.

Currently, for testing of device safety and hemocompatibility, determination of the effect of EC is mostly restricted to end-stage parameters such as hemolysis and thrombosis, and testing of hemolysis is the only mandatory condition. Relatively few studies have addressed the effects of EC on RBC structure, function and life span as probable causes of anemia and RBC-related pathophysiology. Related changes in cell shape, deformability and the appearance of immunological removal signals occur during physiological aging in vivo and during storage in the blood bank. These changes not only affect RBC survival and function, but also render RBCs more susceptible to physiological and pathological stress. Therefore, the general aim of this study was to establish the effect of circulation in a heart-lung machine on RBC homeostasis. In order to distinguish the EC-induced changes and those of the patients’ regulatory reaction, we compared the data obtained from a stand-alone heart-lung machine approach with those obtained during EC-assisted cardiac surgery.

Our results show that: (1) changes in RBC morphology, susceptibility to osmotic stress, deformability, aggregation and the appearance of removal signals increase during circulation in a heart-lung machine, both in vitro and in vivo; (2) the extent and kinetics of these changes are modified by the natural history of the RBCs, in particular the length of storage time in red blood cell concentrates in the blood
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bank; (3) circulation of RBCs with a blood bank storage time of less than one week in a stand-alone heart-lung machine provides a strong system for the improvement of extracorporeal circuit technology and surgery procedures in vivo.

MATERIALS AND METHODS

Red blood cell concentrates

Stored RBCs were obtained as standard RBC concentrates from the regional blood bank Sanquin Bank South East Region, Nijmegen, The Netherlands. At each storage time point, RBCs from three concentrates were pooled in order to reduce interindividual variability, and washed three times using Ringer solution (125 mM NaCl, 5 mM KCl, 1 mM MgSO4, 2.5 mM CaCl2, 5 mM glucose, 32 mM HEPES, pH 7.4) by repeated centrifugation (10 min, 1500g).

Stand-alone extracorporeal circuit

The extracorporeal circuit was designed to mimic a clinical cardiopulmonary bypass circuit and consisted of an hard shell venous reservoir (Inspire HVR, LivaNova, Mirandola, Italy), 3/8 inch tubing with a phosphorylcholine-based coating (LivaNova), a roller pump, an oxygenator (Inspire 6, LivaNova), an arterial filter (Pall AL6, Terumo Europe, Leuven, Belgium), and an arterial cannula (EOPA 22 Fr, Medtronic, Heerlen, The Netherlands). The priming fluid, identical to that used during surgery, was composed of 1000 ml gelofusin (40 g/L, B. Braun, Melsungen, Germany), 50 ml albumin solution (Albuman 200 g/l, Sanquin Plasma Products, Amsterdam, the Netherlands), 3750 IE unfractionated heparin (LEO Pharma, Amsterdam, the Netherlands), 25 ml sodium bicarbonate (84 g/l, B. Braun), 5 ml calciumgluconate (100 mg/ml, B. Braun) and 100 ml mannitol (150 g/l, Baxter Nederland, Utrecht, the Netherlands). Before the start of the experiments, RBCs were suspended in priming fluid, and the volume of priming fluid in the heart-lung machine was adjusted in order to achieve a starting hematocrit of 25%.

Experimental conditions were identical to those maintained during cardiac surgery, employing a flow rate of 4.5 l/min, a post-oxygenator pressure of 178 mmHg (+/- 2 mmHg) generated by an occluder between arterial filter and arterial cannula, a
fraction of inspired oxygen of 21%, a CO\textsubscript{2} flow of 4 ml/min, a sweep gas flow of 1000 ml/min and a fluid temperature of 36.7 ± 0.1°C.

**Cardiac surgery**

The study protocol followed the guidelines of the declaration of Helsinki, and was approved by the local ethics committee (‘Commissie Mensgebonden Onderzoek regio Arnhem-Nijmegen’, The Netherlands; file number 2018-4421). Informed consent was obtained from patients scheduled for elective coronary artery bypass grafting, using both the left internal thoracic artery and a saphenous vena as conduits. Exclusion criteria were a body surface area < 2.0 m\textsuperscript{2}, age > 75 yr, preoperative hematocrit < 30%, application of normovolemic hemodilution and peroperative red blood cell (RBC) transfusion.

The semi-closed extracorporeal system used for these procedures included a soft-shell venous reservoir (VBR 1900, Getinge, Hilversum, The Netherlands), a centrifugal pump (Revolution, LivaNova), a hollow fibre oxygenator (Quadrox-i adult, Getinge), an arterial filter (PALL AL6), a hard shell cardiotomy reservoir (VHK 71000, Getinge) and coated tubing (Bioline, Getinge). Standard cannulas were used: arterial 22 Fr DLP (Medtronic) and venous dualstage 36/46 Fr (Edwards, Dilbeek, Belgium). Priming fluid was the same as described above, and 1200 ml was mixed with the patients’ blood. The average volume of the re-transfused cellsaver blood (Xtra, LivaNova) at the end of the procedure was 533 ± 89 ml (N=5).

Anesthesia was performed according to local guidelines. After premedication with oral paracetamol and midazolam, anesthesia was induced with sufentanil, midazolam, propofol, rocuronium and maintained with sufentanil and midazolam. Just before and during the procedure, patients were given tranexamic acid with a bolus of 1000 mg in 15 minutes and continuously 400 mg/hr. Patients were treated with the antibiotic cefazoline 2000 mg (Eurocept, Ankeveen, The Netherlands) administered 60-15 minutes before incision, which was repeated after 4 hours of surgery or when more than 500 ml cell salvage product was returned to the patient. Surgery was performed according to local guidelines. In brief, patients were heparinized using an initial dose of 300 IU/kg unfractionated heparin (LEO Pharma), and during surgery the activated clotting time was maintained at > 480 seconds.
Patients were mildly cooled (> 35°C nasal temperature) and rewarmed before weaning from the heart-lung machine (nasal temperature >36.5°C, peripheral temperature >35°C). Targeted flow rates during cardiopulmonary bypass were 2.6 l/min/m², yielding a venous saturation of 70-80%. The average cardiopulmonary bypass time was 87 ± 31 minutes (N=5). When needed, sevoflurane and noradrenalin were administered to maintain mean arterial bloodpressures of 50-60 mm Hg. Warm blood cardioplegia according to Calafiori was used to arrest the heart, and was repeated every 15-25 minutes. Protamin hydrochloride (Meda Pharma, Amstelveen, The Netherlands) in a 1:1 ratio with administered heparin neutralized residual heparin activity following surgery. After weaning from the heart-lung machine, residual blood in the extracorporeal circuit was processed by autotransfusion system and immediately returned to the patient.

**RBC analysis**

Samples of five ml were taken pre-surgery, immediately before starting the heart-lung machine and subsequently every 30 minutes, before aortic clamping, before removing the aortic clamp, before weaning from the heart-lung machine, and before leaving the operation room. RBCs were isolated by centrifugation (5 min, 400g). The degree of hemolysis was estimated by measuring the absorbance of the supernatant at 415 nm.

Semi-quantitative microscopic analysis and cell morphology classification of RBCs into discocytes, echinocytes, stomatocytes etc. was performed as described before.234

The percentage of phosphatidylserine (PS)-exposing RBCs was determined using Annexin V, essentially as described before.150 One million RBCs were incubated with phycoerythrin (PE)-labelled Annexin V (1:25, BD Pharmingen, Hoeven, the Netherlands) for 20 min at room temperature in the dark. Tert-butylhydroperoxide-treated RBCs (1mM, 45 min at room temperature) served as positive controls.

Deformability and aggregation were measured using a laser-assisted optical rotational cell analyser (Lorrca MaxSis, Mechatronics, The Zwaag, the Netherlands) as described previously.108 Osmotic gradient ektacytometry, i.e. RBC deformability during exposure to a gradient of increasing osmolality, was performed using the
osmoscan module of the Lorrc a ektacytometer, as previously described108. Osmotic fragility was also assessed by measuring the free hemoglobin concentration at 415 nm after incubation of the RBCs in various NaCl concentrations (150, 125, 100, 75, 50 and 0 mM)235.

Microvesicles were isolated from the supernatants obtained after centrifugation of RBC samples (5 min, 400g) and analysed by flow cytometry as described before221, using a FITC-labelled monoclonal anti-glycophorin A antibody (1:100, clone 11E4B-7-6, Beckman Coulter, Fullerton, CA, USA) to identify RBC-derived microvesicles, and Annexin V-PE (1:25, BD Pharmingen, Hoeven, the Netherlands) to evaluate their PS exposure.

Statistical analysis

Differences between two groups were determined using a paired t-test. Differences between three groups and time points were determined by repeated one-way ANOVA in combination with Tukey’s post-test. Two-sided p-values were used to determine statistical significance when p < 0.05.

RESULTS

RBC volume and morphology

Flow cytometry enables the identification of changes in cell volume, using forward scatter236. One-week old RBCs showed an initial, transient decrease in forward scatter, which was followed by an increase beyond the values obtained during the first two hours. Three-week old RBCs showed a steady increase up to 2.5 hours of circulation, followed by a small decrease. Five-week old RBCs showed a slight decrease in forward scatter throughout the circulation time (Figure 4.1A). The RBCs from patients undergoing EC during cardiac surgery showed changes that were similar to those of one week-stored RBCs, namely an initial decrease in forward scatter followed by an increase towards the starting value, although there still was a significant difference between the forward scatter after and before surgery (Figure 4.1B).
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Figure 4.1: RBC forward scatter before, during and after extracorporeal circulation.

A, forward scatter of one, three and five weeks old RBCs during circulation in the heart-lung machine. For each storage period, RBCs of three different donors were pooled, as described in Materials and Methods. Data points are the mean of three independent measurements of three pooled RBC concentrates (SD not shown; ≤ 14 FSC units for all data points; 95% CI: 1-week old: 458.1-466.6; 3-weeks old: 460.9-469.1; 5-weeks old: 469.9-474.7); B, forward scatter of RBCs from five patients who underwent coronary bypass surgery with extracorporeal circulation. The different symbols represent the individual patients, with the confidence limits (95% CI). Forward scatter is expressed as the median of the height of the forward scatter peak obtained by flow cytometry. All differences in the forward scatter between BC and 4 hours in panel A, and the differences between BC and AC in panel B are statistically significant (p<0.05), as are the differences between one-week old, three-week old and five-week old RBCs. BC, before circulation; DC, during circulation; AC, after circulation. For details see Materials and Methods; *, p<0.05.

Storage in the blood bank is accompanied by changes in RBC morphology from discocytes to echinocytes, and from echinocytes to irreversibly misshapen, spherocytic RBCs. Especially the appearance of the latter is accompanied by a decrease in ATP production, in deformability and in cell membrane content.

Circulation in the heart-lung machine had little impact on cell morphology. Interestingly, only the one-week old RBCs underwent a time-dependent decrease in discocytes that was accompanied by an increase in echinocytes in the first hour, followed by an increase in misshapen cells. RBCs that had been stored in the blood bank for three and five weeks contained a relatively high fraction of misshapen cells, but this hardly changed during circulation in the heart-lung machine (data not shown).
Osmotic fragility

Exposure to changes in osmotic conditions is a stressor that is associated with EC and that may affect RBC survival\textsuperscript{151}. The ability to withstand hypo-osmotic conditions decreased with storage time (Figure 4.2A), as reported before\textsuperscript{151,235,239}. In the heart-lung machine, the ability to withstand osmotic changes increased during the first hours of circulation. Interestingly, after approximately two hours in the heart-lung machine, the one-week old RBCs became more susceptible to lysis, but they did remain more resistant to hypo-osmotic stress than the three-week and five-week old RBCs (Figure 4.2A). The RBCs from patients undergoing surgery underwent small changes in osmotic fragility that resembled those of one-week old cells (Figure 4.2B).

**Figure 4.2: Osmotic fragility of RBCs during extracorporeal circulation**

A, osmotic fragility of stored RBCs measured as absorption of cell-free hemoglobin at 415 nm after incubation of RBCs in 100 mM NaCl. All data are the mean of three independent measurements of three pooled RBC concentrates (SD ≤ 0.007 absorbance units; 95% CI: 1-week old: 0.264-0.419; 3-weeks old: 0.564-0.764; 5-weeks old: 0.793-1.106); B, osmotic fragility of RBCs from five patients who underwent cardiac bypass surgery with extracorporeal circulation with confidence limits (95% CI), as described in the legend of Figure 4.1 and Materials and Methods. All data were extracted from RBC fragility curves. In panel A, the values of one-week, three-weeks and five-weeks old RBC are significantly different from each other for all time points (*, \(p < 0.001\)). The values of the patients’ RBCs (panel B) did not differ significantly from each other.
**Deformability**

Deformability is required for capillary passage, and compromised deformability is associated with decreased oxygen transport and RBC life span\textsuperscript{108}. Circulation in the heart-lung machine induced a decrease in the deformability of all blood bank RBCs (Figure 4.3A), whereas the deformability of the patients’ RBCs did not change during surgery (Figure 4.3B). The difference between the starting values is probably due to the change in cell volume inherent to blood bank storage\textsuperscript{237}, and is also the most likely explanation for the differences between one-week old RBCs and the patients’ RBCs in deformability under various osmotic conditions (Supplementary Figure 4.S1). On the other hand, both the aggregation index, a parameter for the tendency to form aggregates, and the threshold shear stress, an indication for the strength of the aggregates, increased to a stable level during EC-assisted surgery (Figures 4.3C and 4.3D). The decrease in aggregability of the patients’ RBCs at the start of the extracorporeal circulation (Figure 4.3C) is probably due to the priming fluid-induced change in physical conditions, i.e. the concentration of fibrinogen.
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Figure 4.3: The effect of storage and circulation time on RBC deformability and aggregation.

A. Maximal deformability, expressed as elongation index, of one-week and five-week old RBCs during circulation in a heart-lung machine. The standard deviation of all values was less than 0.01 EI units (95% CI: 1-week old: 0.690-0.711; 5-weeks old: 0.698-0.714); B, Deformability of the patients’ RBCs during extracorporeal circulation-assisted cardiac surgery + confidence interval (95% CI); C, Aggregation Index of patients’ RBCs with confidence interval (95% CI); D, the minimal shear rate needed to prevent aggregation of the patients’ RBCs + confidence limits (95% CI). The data are the mean of four donors. In panel A, the values of five-week old are significantly different from each other for all time points, as are the differences in threshold shear rate between one hour of EC-assisted surgery and after surgery in panel D (*, p < 0.01).
Phosphatidylinerse exposure

The presence of phosphatidylinerse (PS) in the outer leaflet of the RBC membrane results from loss of the asymmetric phospholipid distribution, and indicates a disturbance of cell homeostasis. An increase in exposed PS, which is often associated with changes in RBC morphology, contributes to recognition and removal by macrophages. As observed before, during storage the fraction of PS-exposing cells increased with storage time, after three weeks of storage. Also, the fraction of PS-exposing RBCs from the blood bank increased during circulation in the heart-lung machine, which was followed by a small decrease, except for the five-week old RBCs (Figure 4.4A). The patients’ RBCs showed a similar pattern, i.e. an initial increase followed by a small decrease (Figure 4.4B).

![Figure 4.4: Exposure of phosphatidylinerse during circulation in a heart-lung machine.](image)

A. Percentage of PS-exposing RBCs in one-week, three-week and five-week old RBC concentrates during circulation in the heart-lung machine. For each storage period, RBCs of three different donors were pooled, as described in Materials and Methods. The data represent the mean value of three representative measurements of at least 200,000 cells/measurement. (SD ≤ 0.02 %; 95% CI: 1-week old: 0.180-0.265; 3-weeks old: 0.214-0.285; 5-weeks old: 0.238-0.388); B, fraction of PS-exposing RBC of five patients who underwent extracorporeal circulation-assisted cardiac bypass surgery with confidence limits (95% CI). In panel A, the values of one- and three-week old RBC are significantly different from each other for all time points (*, p < 0.05). The values of the patients’ RBCs (panel B) did not differ significantly from each other.
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Vesiculation

Formation of microvesicles (MVs) is an integral part of the aging process of the RBC in vivo and in vitro, and is associated with decreased RBC deformability, and immunological recognition and removal\(^{50,166,167,240,241}\). The five-week old red blood cell blood bank concentrates contained almost fifteen-fold increased concentrations of vesicles compared with one-week and three-week old RBC concentrates, confirming previous observations\(^{151}\). Throughout the circulation time, the blood bank RBCs produced increasing numbers of microvesicles, especially after three hours of circulation (Figure 4.5A). In contrast, the microvesicle concentration in the patients’ blood was much lower, and this did not increase but decreased after the first hour of circulation/surgery time (Figure 4.5B), probably due to rapid immune recognition and removal\(^{150,165}\).

![Figure 4.5: Microvesicle generation of RBCs during circulation in a heart-lung machine.](image)

A, Microvesicle concentration of one-week, three-week and five-week old RBCs generated during circulation in the heart-lung machine (95% CI: 1-week old: 2.4-9.6 x 10\(^3\); 3-weeks old: 1.2-3.1 x 10\(^3\); 5-weeks old: 1.5-8.5 x 10\(^3\)). For each storage period, RBCs of three different donors were pooled, as described in Materials and Methods; B, microvesicle concentration of plasma of five patients during extracorporeal circulation-assisted cardiac surgery + confidence limits (95% CI). Microvesicles were isolated, quantitated, and characterized as described before (30). In panel A, the values of one-week old RBC derived microvesicles are significantly different from each other for all time points (*, \(p < 0.05\)). The values of the patients’ RBCs (panel B) did not differ significantly from each other.

It has been suggested that storage-associated susceptibility to oxidative stress might affect RBC structure and function of RBCs in an extracorporeal circulatory system\(^{233}\), and oxidation in vitro induces vesiculation as well as PS exposure\(^{151}\). We found an increase in methemoglobin concentration in one-week old RBCs during
circulation in the stand-alone heart-lung machine, as well as a temporary increase in methemoglobin in the patients’ RBCs during surgery (Supplementary Figure 4.52), supporting this suggestion. The methemoglobin concentration followed the same pattern as the microvesicle concentration in the patients’ blood. The values of these parameters showed a positive correlation ($r$, 0.639; $p<0.05$; $N=17$).

**DISCUSSION**

During circulation through the heart-lung machine, RBCs are exposed to an environment that is very different from the circulation in vivo. The prolonged immersion of RBCs in a non-physiological solution, interaction with artificial surfaces and turbulence all induce structural alterations at the molecular level that are likely to affect their biophysical, biochemical and immunological properties130. In addition, EC may be used in combination with transfusion of stored RBC concentrates, containing RBCs that have undergone blood bank procedures as well as storage time-associated, aging-related changes in structure and function242. Even though heart-lung machines meet criteria for RBC integrity, surgery involving extracorporeal circulation is associated with side effects that may be related to changes in RBC function and survival243. Here we show that the conditions imposed by circulation of RBCs through a heart-lung machine induce small, but biologically relevant changes in RBC structure and function. The kinetics of most changes showed a biphasic pattern, in which the early phase may represent the response of the fraction of the oldest and probably most vulnerable cells of the population. The duration of this phase increased with RBC storage time, probably because the size of the most vulnerable fraction increases with time spent in the blood bank. The same phenomenon may be responsible for the fast removal of a considerable fraction of the RBCs after transfusion151,237. Since aging in vivo is also associated with an increased susceptibility to stress244, the oldest, most susceptible autologous RBCs may disappear quickly from the circulation of patients undergoing heart-lung machine-assisted cardiac surgery. Also, the combination of mechanical stress with the surgery-associated changes in RBC environment may induce changes in membrane organization that cause the observed changes in deformability and aggregation (Figure 4.3; see also ref. 227), and/or the appearance of removal signals such as PS leading to phagocytosis (Figure 4.4). Although the changes in
aggregation behavior we observed for the RBCs of patients undergoing EC-assisted surgery were observed for a relatively short period of time (Figure 4.3), others have described much longer-lasting effects. We postulate that the later changes, including the increase in threshold shear rate reported before, reflect the response of the remaining, more resilient RBCs. It has been suggested that oxidation, together with storage time, may be one of the stress factors. Our findings on the changes in methemoglobin content in stored RBCs during circulation in a stand-alone heart-lung machine, and in the patients’ RBCs during surgery (Supplementary Figure 4.S2), support this suggestion.

The changes in cell volume, osmotic fragility and deformability of the RBCs that had been stored in the blood bank for one week, indicate that changes in membrane organization that affect mechanisms involved in transport of water and/or ions, occur mainly in the first hours of circulation in the heart-lung machine. Later changes, especially the observed decrease in maximal deformability (Figure 4.3), are mainly due to a decrease in the surface area/volume ratio. The concomitant increase in microvesicle numbers (Figure 4.5) suggests that the latter is caused by a loss of membrane. The differences between the stand-alone and the patient data are likely to be due to the fast removal of microvesicles - and PS-exposing RBCs - from the patients’ circulation. Importantly, our results show that circulation in a heart-lung machine has an impact on RBC structure and function that extends well beyond hemolysis. Patients on extracorporeal life support who are dependent on extracorporeal oxygenation and/or circulatory support for weeks may be even more affected by the alterations in RBC structure and function reported here.
CONCLUSIONS

The similarity of the data from our stand-alone setup, obtained with blood bank RBCs, with those observed in the patients’ RBCs during extracorporeal bypass-assisted surgery, show that a stand-alone extracorporeal circulation device, in combination with RBCs that have been stored in the blood bank for not longer than one week, mimics many subtle alterations in structure and function of the RBCs in vivo in patients undergoing cardiopulmonary bypass surgery. Thus, these findings provide a biophysical, biochemical and immunological system for validation and improvement of extracorporeal and another flow-assist device technology.\textsuperscript{226,246}

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DISCLOSURE

The authors declare no conflict of interest.

SUMMARY OF AUTHOR CONTRIBUTIONS

JKFL, HV, LvG, RB and GB designed the study; JKFL, DL, and HV collected the data, performed the assays and the primary analyses; all authors contributed to the interpretation of the data, and to the various drafts and final version of the manuscript.
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SUPPLEMENTARY MATERIAL

Supplementary Figure 4.S1: The effect of circulation time on RBC deformability at various osmotic conditions as measured with osmotic gradient ektacytometry (osmoscan).

A, Osmoscans of one-week old RBC during circulation in a stand-alone heart-lung machine; B, Representative osmoscans of the RBCs from one patient during heart-lung machine-assisted cardiac surgery.
Supplementary Figure 4.52: The effect of storage and circulation time on methemoglobin formation.

A, Methemoglobin concentration of stored RBCs during circulation relative to the value before the start of the circulation (95% CI: 1-week old: 0.171-0.191; 3-weeks old: 0.152-0.242; 5-weeks old: 0.153-0.222; B, Methemoglobin concentration of the patients’ RBCs during heart-lung machine-assisted cardiac surgery + confidence limits (95% CI). BC, before circulation; DC, during circulation; AC, after circulation.

In panel A, the values of three- and five-week old RBC are significantly different from each other for all time points (*, p < 0.05). The values of the patients’ RBCs (panel B) did not differ significantly from each other.
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Red Blood Cell Homeostasis and Altered Vesicle Formation in Patients with Paroxysmal Nocturnal Hemoglobinuria

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How RBC homeostasis is affected by pathology

Chapter 5

How RBC homeostasis is affected by pathology
A subset of the red blood cells (RBCs) of patients with paroxysmal nocturnal hemoglobinuria (PNH) lacks GPI-anchored proteins. Some of these proteins, such as CD59, inhibit complement activation and protect against complement-mediated lysis. This pathology thus provides the possibility to explore the involvement of complement in red blood cell homeostasis and the role of GPI-anchored proteins in the generation of microvesicles (MVs) \textit{in vivo}. Detailed analysis of morphology, volume, and density of red blood cells with various CD59 expression levels from patients with PNH did not provide indications for a major aberration of the red blood cell aging process in patients with PNH. However, our data indicate that the absence of GPI-anchored membrane proteins affects the composition of red blood cell-derived microvesicles, as well as the composition and concentration of platelet-derived vesicles. These data open the way toward a better understanding on the pathophysiological mechanism of PNH and thereby to the development of new treatment strategies.
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INTRODUCTION

Paroxysmal nocturnal hemoglobinuria (PNH) is a highly debilitating disease that is characterized by intravascular hemolysis, arterial, and venous thrombosis and a variety of symptoms related to smooth muscle dystonia. PNH is a rare disease with an incidence of 1–2 per 1,000,000 persons per year and is frequently associated with bone marrow failure such as aplastic anemia. PNH is caused by clonal expansion of multipotent hematopoietic stem cells with somatic mutations in the PIGA gene. PIGA encodes for an enzyme that is critical in the synthesis of the first intermediate in the pathway of glycosylphosphatidylinositol (GPI) anchors. As a consequence, the absence of PIGA activity results in hematopoietic cells that are deficient in GPI-anchored proteins. In RBCs, the absence of the GPI-anchored proteins decay-accelerating factor (DAF; CD55) and membrane inhibitor of reactive lysis (MIRL; CD59) that protect against complement-mediated lysis renders red blood cells (RBCs) highly vulnerable to intravascular hemolysis. This results not only in anemia but also in the release of free hemoglobin and iron, which catalyzes the generation of reactive oxygen species and subsequent NO depletion and vasoconstriction. For untreated patients, thrombosis is the most common cause of death.

The monoclonal antibody eculizumab is the most effective drug used in PNH. Eculizumab blocks the cleavage of C5 by the C5 convertase into C5b and thereby inhibits the formation of the terminal membrane attack complex (MAC) C5b-9 and consequent hemolysis of abnormal RBCs. This reduces RBC destruction and transfusion requirements. Nevertheless, the opsonizing effects of activated complement factors such as C3d may induce RBC phagocytosis. At present, the mechanism(s) responsible for clonal expansion during hematopoiesis and the variable clinical manifestations of the disease have only partially been elucidated, but increased removal of RBC may contribute to the pathophysiology of PNH. RBC homeostasis is dependent on the generation of young and removal of aged RBCs. The latter process is initiated by binding of senescent cell-specific IgG, the appearance of molecules that may trigger pathological reactions, such as immunoreactive epitopes on damaged membrane proteins, and exposure of phosphatidylserine (PS) in the outer leaflet of the lipid bilayer, all leading to phagocytosis.
proteomic, and metabolomic studies, a molecular picture of the pathways involved in the normal aging and removal process of RBCs has emerged: oxidative damage-induced, high-affinity binding of hemoglobin to the cytoplasmic domain of band 3, activation of Ca²⁺-permeable channels, phosphorylation-controlled alterations in morphology and metabolism affecting ATP production and redox status, degradation of band 3 and/or aggregation of band 3 fragments, binding of IgG, and microvesicle (MV) generation. Physiological anti-band 3 IgG has been reported to have a high affinity for dimeric C3b, thereby linking RBC phagocytosis to complement activation.

During physiological RBC aging, there is a small decrease in the content of GPI-anchored DAF and MIRL, and in the content and activity of acetylcholinesterase (AChE), another GPI-anchored protein. The latter observation suggests that the activities of DAF and/or MIRL might also decrease in healthy individuals and thereby contribute to complement-mediated opsonization and removal of old RBCs. AChE is increased in microvesicles, suggesting that changes in the distribution of GPI-anchored proteins in microdomains are associated with microvesicle (MV) generation. As a consequence, the absence of GPI-anchored proteins may affect the microvesiculation process. Indeed, some data indicate that microvesiculation of RBCs and platelets may be impaired in PNH patients. Also, it has been shown that activated complement induces the massive formation of vesicles with a strong pro-coagulant activity. Thus, the absence of GPI-anchored proteins may have a pronounced effect on RBC morphology, function, and survival. In addition, exposure of the pro-coagulant and removal signal PS, which is in general associated with abnormal membrane organization and vesiculation in damaged or stressed, but not in aged RBCs, has been reported to be increased in RBCs of PNH patients.

Here, we have selected a number of aging-associated parameters from this current knowledge of the molecular mechanisms involved in physiological RBC homeostasis that might be relevant for the pathophysiology of PNH, in order to explore the effect of the absence of GPI-linked proteins on RBC structure, function, aging, and removal in vivo. Our data, obtained from PNH patients with various clone sizes and following various treatment regimes, indicate no significant effects of the absence of GPI-linked proteins on RBC turnover but emphasize the
heuristic value of more, detailed studies on the origin, composition, and activity of RBC-derived and platelet-derived microvesicles.

**MATERIALS AND METHODS**

**Red Blood Cell Sampling**

Blood was collected by venipuncture from healthy volunteers and 15 patients after obtaining written informed consent, and using EDTA as anticoagulant, following the guidelines of the local medical ethical committee (CMO regio Amhem Nijmegen) and in accordance with the Declaration of Helsinki. Leukocytes and platelets were removed as described before using Ficoll-Paque. The time between blood collection, fractionation, and analysis was identical for all samples.

**Red Blood Cell Fractionation and Microscopic Analysis**

RBCs were fractionated according to cell density using discontinuous Percoll gradients ranging from 40% Percoll (1.060 g/ml) to 80% Percoll (1.096 g/ml) as described before. The various RBC fractions were isolated and washed three times with Ringer's solution by repeated centrifugation for 5 min at 400 g before analysis. RBC morphology was analysed using a TCS SP5 confocal laser scanning microscope (Leica Microsystems, Mannheim, Germany) as described before.

**Isolation and Characterization of Microvesicles From Plasma**

Microvesicles (MVs) were isolated from the platelet-rich plasma (PRP) obtained after differential centrifugation as described before.

**Flow Cytometry Analysis**

Classification of the RBCs according to PNH type was performed by flow cytometry using FITC-labeled CD235a (clone KC16, 1:100, Beckman Coulter, Fullerton, CA, USA) and PE-labeled CD59 (clone MEM43, 1:400, IQ products, Groningen, the Netherlands) as described before. PNH RBCs were classified based on CD59
content in type III (complete GPI-deficiency), type II (partial GPI-deficiency), and type I (normal expression) cells (Sutherland et al., 2015). APC-labeled CD71 (clone CY1G4, 1:200, Biolegend, San Diego, California, USA) was combined with PE-labeled CD59 to evaluate the percentage of reticulocytes per PNH type. FITC-labeled anti-C3c (1:200, Abcam, Cambridge, UK) and APC-labeled anti-C3d (1 μg/million cells, Assay Pro, St. Louis, Missouri, USA) were combined with PE-labeled CD59 to evaluate the degree of opsonization per PNH type. Staining of band 3 with eosin-5′ maleimide (EMA, Thermo Fisher Scientific, Landsmeer, the Netherlands) was performed by incubating 1 million RBCs with 25 μl of EMA (0.5 mg/ml in Ringer’s solution) in the dark at RT for 15 min. After staining, RBCs were washed three times with Ringer’s solution and analysed by flow cytometry [FACSCalibur instrument (BD Biosciences, Franklin Lakes NJ, USA)] using CELLQuest software (BD Biosciences). Data were analysed with FlowJo cell analysis software v.10 (FlowJo, LLC, Ashland, OR) using 200,000 events. Microvesicle analysis was performed using mixtures of PE-labeled CD59 (1:400), FITC-labeled CD235a (1:100), and PE/Cy5-labeled CD41 (1:10) by flow cytometry as previously described. Sulfate latex microspheres (0.9 μm, Invitrogen, Carlsbad CA, USA) and washed Flow-Count calibration beads (Beckman Coulter, Brea CA, USA) were used for quantification. Microvesicles were classified based on CD59 positivity in CD59-negative (complete GPI-deficiency), low CD59 (partial GPI-deficiency), and wild type (normal expression).

Comparisons and Statistical Analyses

The exclusion criteria for the PNH patients were other hematological comorbidities besides aplastic anemia and having received a red blood cell transfusion within a period of 3 months before analysis. For most analyses, we compared PNH patients with control donors and PNH patients being treated with eculizumab with patients without eculizumab. Differences between groups were determined using a two-way ANOVA test. Non-parametric t-tests or one-way ANOVA tests were used to analyse differences between control and PNH samples. Wilcoxon matched pair tests were used to analyse differences between the various RBC fractions inside the groups, and the Fisher LSD test was used to compare controls and patient samples. Two-sided p’s less than 0.05 were used to determine statistical significance. Relations
between the various parameters were estimated using the Pearson correlation coefficient.

RESULTS

RBC Morphology and Phenotype

During aging in vivo and in vitro, RBCs undergo a series of morphological changes that result in the appearance of deformed, mostly spherocytic cells. Semi-quantitative analysis of these changes has been shown to be informative on RBC hemostasis and on the relationship between morphology, deformability, and survival\(^{234}\). Microscopic analysis of RBCs from patients with PNH showed a tendency to a decrease in the numbers of cells with the regular discocyte form and a concomitant increase in the numbers of echinocyte-like and otherwise misshapen cells, especially in the densest cell fractions (Figure 5.1A). The majority of the patients’ RBCs were type I according to CD59 expression levels (Figure 5.1B), and we found no differences in the percentages of type II and type III cells between the various Percoll layers (Figure 5.1C). Treatment with eculizumab did not result in significant differences in CD59-deficient cells (Figure 5.1D).
Figure 5.1: RBCs morphology and phenotype of PNH patients.

(A) Percentage of discocytes per Percoll fraction in PNH patients (N = 5) and healthy control donors (N = 5); (B) bright field and fluorescence images of anti-CD59-Alexa 647 stained RBCs from a healthy subject and a PNH patient, showing CD59 density; (C) RBCs of PNH patients (N = 9) were separated according to density and analysed by flow cytometry regarding their CD59 content (type I, II, and III); (D) RBCs of PNH patients being treated with eculizumab (T; N = 5) and non-treated PNH patients (NT; N = 4) separated according to density and analysed by flow cytometry according to their CD59 content (type I, II, and III). # Significantly different from type I in the same Percoll fraction (p < 0.05). U, unseparated; f1, f3, f5, fractions of increasing density isolated by Percoll density separation (Materials and Methods).
Membrane/Band 3 Content (Eosine 5′-Maleimide)

RBC aging is accompanied by changes in membrane organization that are associated with the appearance of removal signals and with the loss of cell membrane. Especially, changes in the integral membrane protein band 3 play a pivotal role in the generation of senescence-specific antigens, in the interaction between lipid bilayer and cytoskeleton, and in the generation of 13,50,167,241. The amount of binding of the band 3 probe eosine 5′-maleimide (EMA) is mostly a sensitive marker of band 3 content, but also of Rh, Rh glycoprotein, and CD47, and/or of the loss of membrane268,270. Flow cytometric analysis of the binding of EMA showed a higher EMA signal in all RBC fractions from two different PNH patients tested, independent of cell density and treatment (Figure 5.2). There was no significant difference in the density- associated decrease between control donors or any of the PNH patients. Also, there was no statistically significant correlation between EMA fluorescence and the RBC size (forward scatter) in the RBC fractions of controls and PNH patients taken together ($r = 0.31, p = 0.18, N = 20$).

![Figure 5.2: Eosin 5′-maleimide Mean Fluorescence Intensity (MFI) of RBC fractions.](image)

(A) RBCs of PNH patients ($N = 2$) and of control healthy donors ($N = 7$) of various Percoll fractions were stained with eosin 5′-maleimide (EMA). The degree of staining is expressed as the mean fluorescence intensity (MFI). (B) EMA MFI of RBCs of a PNH patient being treated with (T) and without (NT) eculizumab, separated according to density. Ctrl, healthy donors ($N = 7$). The samples were analysed as described before (see Materials and Methods). *Significantly different from control ($p < 0.05$). U, unseparated; f1, f3, f5, fractions of increasing density isolated by Percoll density separation (Materials and Methods).
Complement Deposition (C3c and C3d)

Activation of complement may lead to deposition of complement fragments on RBC through the CR1 receptor, and the presence of C3b fragments induces phagocytosis of eculizumab-treated, CD59-negative RBCs in vitro. We therefore also probed for the presence of C3c and C3d in density-separated RBCs. For both proteins, we observed a tendency to an increase in the percentage of positive cells with cell density (Figure 5.3). Thus, the content of RBC-bound C3c as well as C3d may increase with cell age, also on type I RBCs with a normal content of CD59 (Figure 5.3). These findings are in agreement with previous indications for the involvement of complement in phagocytosis in vitro. We found no significant correlations between these parameters and treatment with eculizumab (data not shown).

Figure 5.3: Complement deposition on density-separated RBCs.

(A) Percentage of C3c-positive RBCs in the PNH RBC population divided per CD59 content (type I, II, and III) per density (Percoll fraction; N = 2); (B) percentage of C3d-positive RBCs in the PNH RBC population according to CD59 content (type I, II, and III) per Percoll fraction (type I, II, and III; N = 3). The samples were analysed as described before (see Materials and Methods). U, unseparated; f1, f3, f5, fractions of increasing density isolated by Percoll density separation (Materials and Methods).

Reticulocytes

Aberrant RBC structure resulting in a decreased mean life and leading to anemia is, in many cases, compensated by increased erythropoiesis, as indicated by changes in the size of the reticulocyte fraction. The hematological data show a large variability in the size of the reticulocyte fractions of our patients, without any
significant correlation with other patient variables, although most eculizumab-treated patients had higher reticulocyte numbers than the patients without eculizumab (Supplementary Table 5.1). Flow cytometric analysis of the RBCs of a few PNH patients showed similar data, also without significant differences between donors or RBC fractions (Figures 5.4 A, B). In general, most reticulocytes were found in the lightest density fractions upon Percoll separation, i.e., fraction 1 (Figure 5.4A), as shown before for healthy individuals50. The fraction of type III, CD59-lacking reticulocytes was considerably higher than the other types (Figure 5.4C), which may reflect a disturbed differentiation and/or maturation process in the absence of GPI-linked proteins265.

Figure 5.4: Reticulocytes in patients with PNH.

(A) Percentage of CD71-expressing RBCs from the blood of PNH patients (N = 2) and healthy control donors RBCs (N = 4) of various Percoll fractions after staining with APC-labeled CD71; (B) percentage of APC-CD71-positive RBCs of a PNH patient being treated with eculizumab (T), a non-treated PNH patient (NT), and healthy control donors in the reticulocyte-enriched Percoll fraction 1 (Ctrl; N = 4); (C) percentage of APC-CD71-positive RBCs in the PNH RBC population per CD59 content (type I, II, and III) in fraction 1 (N = 2). The samples were analysed as described before (see Materials and Methods). U, unseparated; f1, f3, f5, fractions of increasing density isolated by Percoll density separation (Materials and Methods).
Microvesicles

Microvesicle generation is an integral part of the physiological RBC aging process, and changes in microvesicle concentration as well as composition occur in patients with disturbed RBC homeostasis\textsuperscript{241}. We found no significant differences in the concentrations of RBC-derived microvesicles between PNH patients and controls (Figure 5.5A). However, the concentration of PS-negative microvesicles in the plasma of PNH patients was higher than in the plasma of control donors (Figure 5.5B). The concentration of CD59-high RBC-derived microvesicles was higher than that of the other types in the plasma of control donors but not in the plasma of PNH patients (Figure 5.5C). Platelet-derived microvesicle concentrations were much higher in the plasma of PNH patients than in controls (Figure 5.5D), both the PS-positive and the PS-negative microvesicles (Figure 5.5E). Remarkably, almost all platelet-derived microvesicles were devoid of CD59, including those from the plasma of control donors (Figure 5.5F). We observed no statistically significant correlations between the numbers of RBC-derived and platelet-derived vesicles ($r = -0.40, p = 0.28, N = 9$).
How RBC homeostasis is affected by pathology

Figure 5.5: Microvesicle numbers and composition in the blood of patients with PNH.

(A) Concentration per microliter (MV/μl) of RBC-derived, CD235a-positive microvesicles in the blood of PNH patients (N = 9) and control healthy donors (N = 6); (B) concentration of RBC-derived microvesicles in the blood of PNH patients (N = 9) and control healthy donors (N = 3), distinguished according to their reactivity to Annexin V (phosphatidylserine-positive (PS+) or negative (PS−)); (C) RBC-derived microvesicles were categorized into wild type, CD59-low and CD59-negative PNH, N = 9; Ctrl, N = 3), as described for RBCs (Materials and Methods); (D) concentration of CD41-positive, platelet-derived microvesicles in the blood of PNH patients (N = 9) and control healthy donors (N = 6); (E) concentration of platelet-derived microvesicles according their reactivity to Annexin V (PS+ or PS−; PNH, N = 9; Ctrl, N = 3); (F) platelet-derived microvesicles were categorized into wild type, CD59-low and CD59-negative as described for RBCs and quantified and analysed by flow cytometry as described before (PNH, N = 9; Ctrl, N = 3). # Significantly different from the other parameter (p < 0.05); *Significantly different from the patients’ samples (p < 0.05).
DISCUSSION

RBC Aging and Generation of Microvesicles

Red blood cells of PNH patients lack the key GPI-anchored membrane proteins that protect against activated complement. We postulated that this change in membrane composition has a more wide-spread effect on membrane organization and thereby on various aspects of RBC homeostasis. The most obvious aspects derive from the role of complement in removal of senescent RBCs and the involvement of GPI-linked proteins in microdomain-associated generation of microvesicles. In this exploratory study, we did not find significant indications for a pronounced alteration of RBC homeostasis in patients with PNH, as based on cell volume, or cell density, and morphology on clinical hematology parameters, including LDH values. Thus, in most of our patients, the lack of GPI-anchored proteins does not seem to cause a major disturbance of the physiological RBC aging mechanisms.

Nevertheless, there were clear differences related to membrane composition and microvesicle formation. The EMA measurements showed significant differences between the RBCs of PNH patients and of control donors. The tendency to a density-associated decrease in EMA staining might be due to loss of band 3 and/or membrane with aging by vesiculation, both in RBCs from control donors and from PNH patients. This has been postulated before for physiological aging in vivo. However, the absence of a statistically significant correlation between EMA fluorescence and the RBC size, based on the cytometer parameter forward scatter, suggests that in the RBCs from PNH patients, the band 3 protein content is not a direct function of cell size. EMA staining is affected by changes in band 3 conformation and membrane organization as well. Combined with the considerable fractions of PS-negative and CD59-lacking microvesicles in the blood of PNH patients, these data indicate that the organization of the RBC membrane, as well as the mechanism of microvesicle generation, are altered by the absence of GPI-linked proteins. This may be a direct effect, but also the consequence of the deposition of C3b. The latter not only affects lateral mobility of CD59 and band 3 molecules but also membrane viscosity and deformability. Our in vivo data support the involvement of GPI-linked proteins in microvesicle formation during RBC aging in vitro. The differences in mechanisms leading to
the generation of microvesicles with and without PS at their outside remain to be established, as well as the effect on biological activity.

Since PS exposure contributes to recognition and removal of microvesicles by macrophages\textsuperscript{161}, its absence may not only affect their pro-coagulant activity but also their lifespan. Fusion between microvesicles and RBCs may underlie the reported transfer between CD55 and CD59 from normal RBCs to RBCs without these proteins\textsuperscript{205}. Thus, microvesicles generated by PNH RBCs may also fuse with normal RBCs, thereby affecting their membrane organization as well. Furthermore, increased levels of RBC-derived microvesicles may affect NO bioavailability\textsuperscript{274} and induce activation of endothelial cells and tissue factor expression\textsuperscript{275}, thereby contributing to the widespread thrombosis in patients with PNH.

**Platelet Microvesicles and Thrombosis**

Platelets without CD59 have been described to catalyze the rate of prothrombin conversion upon treatment with complement C5b-9 in vitro, and this was associated with an increase in microvesicle formation\textsuperscript{276}. RBC-derived and platelet-derived, phosphatidylserine-positive microvesicles have been reported to be increased approximately two-fold in the blood of PNH patients\textsuperscript{183}. We found equal concentrations of RBC-derived microvesicles in the plasma of PNH patients and healthy donors, but much larger RBC-derived, phosphatidylserine-negative microvesicle concentrations in the blood of PNH patients (Figure 5.5B), and larger concentrations of platelet-derived vesicles (Figure 5.5D). In the plasma of eculizumab-treated PNH patients, the numbers of RBC-derived vesicles were lower than in patients who had not been treated with eculizumab (Supplementary Figure 5.S1). The absence of a statistically significant correlation between the concentrations of RBC-derived and platelet-derived microvesicles indicates that the absence of GPI-linked proteins affects microvesicle generation from RBCs and platelets through different mechanisms. Although in control donors, most platelets are CD59-positive\textsuperscript{277}, almost all platelet-derived microvesicles were CD59-negative (Figure 5.5). There were approximately equal concentrations of platelet-derived vesicles with and without PS at their surface (Figure 5.5E). These data strongly suggest that the absence of GPI-linked proteins does not only have a pronounced stimulatory effect on the generation of microvesicles but also on their composition.
The latter may be related to the presence of tissue factor and is likely to affect their function\cite{185}. Our recent finding that platelet-derived microvesicles can prevent differentiation of regulatory T-cells through P-selectin\cite{266} emphasizes their pivotal role in the pathophysiology of many diseases that may include PNH\cite{185}. Although the name suggests otherwise, most platelet-derived microvesicles originate not from platelets, but from megakaryocytes in the bone marrow\cite{278,279}. It is not known how the absence of GPI-linked proteins affects megakaryocyte biology and/or platelet activation. These data support the importance of an extensive characterization of origin, composition, and biological activity of CD41-positive microvesicles. Such studies may help in establishing an urgently needed, robust marker of platelet activation.

CONCLUSION

The heterogeneity of the patient population and the concomitant small numbers available for statistical comparisons of all parameters preclude a robust answer on the question whether RBC aging is altered in patients with PNH. However, the combined results of the selected aging-associated parameters\cite{10,13,167} do not reveal a major aberration of the physiological RBC aging process in patients with PNH. Remarkably, formation of microvesicles by RBCs is altered in patients with PNH. This is likely due to PNH-related differences in membrane organization that is associated with the absence of GPI-linked proteins. The conspicuous lack of phosphatidylserine exposure on many RBC-derived microvesicles in PNH patients may affect their time in the circulation as well as their contribution to hemostasis and thrombosis. In platelets, PNH-related processes seem not only to induce the appearance of large numbers of phosphatidylserine-negative microvesicles but also to cause excessive formation of microvesicles. Future investigations leading to a better understanding of the mechanisms underlying vesiculation, effect of vesiculation on RBC function and survival, and effect of the various microvesicles on thrombosis in patients with PNH may be instrumental in developing new treatment strategies\cite{280}. 
DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the medical ethical committee “CMO-Regio Arnhem Nijmegen”; with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the CMO-Regio Arnhem Nijmegen.

AUTHOR CONTRIBUTIONS

JF performed all measurements, the analyses, and wrote the first version of the manuscript. FP provided the samples, some protocols, and assisted in writing the manuscript. RB, MA-H, and GB contributed to the setup of the study, the interpretation of the data, and the writing of the manuscript.

FUNDING

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CONFLICT OF INTEREST STATEMENT

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.
Supplementary Figure 5.51: Microvesicle numbers and composition in the blood of patients with PNH CD59 level and treatment.

(A) Concentration (MV/μl) of CD235a-positive, RBC-derived microvesicles (MVs) in the blood of PNH patients who did not receive treatment (NT; N = 3), PNH patients who were treated with eculizumab (T; N = 6), and control healthy donors (N = 6); (B) concentration of RBC-derived microvesicles in the blood of PNH patients who did not receive treatment (NT; N = 3), PNH patients who were being treated with eculizumab (T; N = 6) and control donors (N = 6) according their reactivity to Annexin V (phosphatidylserine positive (PS+) or negative (PS−)); (C) RBC-derived microvesicles in the blood of PNH patients who did not receive treatment (NT; N = 3), PNH patients who were being treated with eculizumab (T; N = 6) categorized in wild type, CD59-low and CD59-negative as described for RBCs (Materials and Methods); (D) concentration of platelet-derived microvesicles (CD41-positive) from the blood of PNH patients who did not receive treatment with eculizumab (NT; N = 3), PNH patients who were being treated with eculizumab (T; N = 6) and control healthy donors (N = 6); (E) concentration of platelet-derived microvesicles in the blood of PNH patients who did not receive treatment with eculizumab (NT; N = 3), PNH patients who were being treated with eculizumab (T; N = 6) and control healthy donors (N = 6), according their reactivity to Annexin V (PS+ or PS−); (F) platelet-derived microvesicles from the blood of PNH patients who did not receive treatment with eculizumab (NT; N = 3), PNH patients who were being treated with eculizumab (T; N = 6) were categorized in wild type, low CD59, and CD59-negative as described for RBCs and quantified and analysed by flow cytometry as described before (see Materials and Methods). *Significantly different from the other parameter in the same group (p < 0.05). *Significantly different between groups (p < 0.05)
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AA, PNH patient with aplastic anemia; N, PNH patient without hematological comorbidities; T, in treatment; NT, not in treatment; RBC, red blood cells (×10^12/L); RBC CS (II/III), red blood cell clone size (type II and III); Hb, hemoglobin (g/dl); Ht, hematocrit (%); MCV, mean corpuscular volume (fl); MCH, mean corpuscular hemoglobin (pg); MCHC, mean corpuscular hemoglobin concentration (g/dl); RDW, red blood cell distribution width (%); Retic, reticulocytes (promille); RBC Tr, red blood cell transfusion in the last 3 months; Leuk, Leukocytes (×10^9/L); Gran CS, granulocytes clone size; Plt, platelet (×10^9/L); LDH, lactate dehydrogenase (U/L); −, not available. Reference values for healthy adults: RBC: for men, 4.7–6.1×10^12/L and for women, 4.2–5.4×10^12/L; Hb: for men, 8.5–11 mmol/L and for women, 7.5–10 mmol/dl; Ht: for men, 0.4–0.54 and for women, 0.36–0.46; MCV: 80–96 fl; MCH: 1.7–2.1 fmol; MCHC: 19.3–22.5 mmol/L; RDW: 11.5–14.5%; Retic: 8–26 promille; Leuk: 4.5–11×10^9/L; Plt: 150–400×10^9/L; LDH: 135–225 U/L.
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Red blood cell aging is involved in the early homeostatic response to exercise-associated stress.

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Chapter 6

How RBC homeostasis is affected by exercise
ABSTRACT

The Four Days Marches of Nijmegen in the Netherlands, the world’s largest yearly walking event, constitutes a unique possibility to study the effect of mechanical and biochemical stressors occurring during moderate-intensity exercise on red blood cell homeostasis in vivo. Longitudinal measurements of biophysical, immunological and functional red blood cell characteristics indicate that moderate-intensity exercise induces an early removal of the oldest red blood cells from the circulation. This results in a functionally improved red blood cell population that has a higher deformability and a decreased tendency to aggregate. Our data indicate that the physiological red blood cell aging process maintains homeostasis in times of stress by accelerated aging of the oldest, most vulnerable red blood cells.
INTRODUCTION

Red blood cell (RBC) homeostasis is maintained through removal of aged, functionally compromised RBCs by macrophages, in combination with erythropoietin-regulated RBC production in the bone marrow. RBC aging is associated with removal of damaged membrane proteins by the shedding of vesicles, loss of deformability and oxygen binding, and with the appearance of molecules and/or epitopes that promote recognition and removal by the immune system. The mechanisms underlying functional as well as immunological RBC aging are likely to be triggered by a compromised redox system, which induces an increase in susceptibility to physiological stress\textsuperscript{122,151}. Experimental support for the latter hypothesis is mostly provided by the response of RBCs from healthy donors aged \textit{in vivo} and RBCs from blood bank concentrates aged \textit{in vitro} to various stress factors \textit{in vitro}\textsuperscript{151,244}. Recent findings indicate not only that RBC homeostasis is affected by systemic conditions such as inflammation, but also that altered RBC function and survival may affect organismal homeostasis\textsuperscript{51,98,165,228}.

There is little evidence from experimental data on the validity of this concept in physiological conditions \textit{in vivo}, with the exception of data on RBC-related parameters during exercise. In trained athletes, exercise has been reported to induce hemolysis (e.g. \textsuperscript{281,282}), and this has been postulated to be associated with a shift towards a decrease in mean cell age of the RBC population and with a concomitant functional improvement\textsuperscript{283}. The Four Days Marches of Nijmegen in the Netherlands, the world’s largest yearly voluntary walking event, presents the possibility to study the effect of prolonged, moderate-intensity exercise on various physiological parameters\textsuperscript{284–287}. Recent observations in Four Days Marches participants on iron metabolism and cytokine responses provide clues for mechanisms underlying physiological adaptations in and functional consequences of RBC homeostasis\textsuperscript{286,287}. Here we investigated a panel of hematological, functional and structural characteristics of RBC aging to test the hypothesis that physiological stress \textit{in vivo} during moderate exercise induces the disappearance of the eldest RBCs from the circulation, and that this results in an overall younger, functionally improved RBC population.
MATERIALS AND METHODS

Study population

Blood was collected by venipuncture using EDTA as anticoagulant from 16 healthy volunteers (11 male, 66 ± 4 years, and five female, 65 ± 6 years) who participated in the 2018 edition of the Nijmegen Four Days Marches (http://www.4daagse.nl/en/), and who were included using the criteria described before (284). The participants walked 30 kilometers (N=8), 40 kilometers (N=7), or 50 kilometers (N=1) on four consecutive days. All participants gave written informed consent. The study was approved by the local Medical Ethical Committee (CMO Arnhem-Nijmegen; 2007-148) and was in accordance with the Declaration of Helsinki. Blood was collected at baseline (day 0), after day 1, in the morning of day 2, and in the afternoon of day 2. Complete analyses were performed on ten participants who donated blood every day.

Plasma, leukocytes and platelets were removed using Ficoll-Paque87. Hemolysis was estimated by measuring the absorbance of the cell-free plasma at 415 nm.

Red blood cell fractionation

RBCs were fractionated according to cell density using discontinuous Percoll gradients ranging from 40% Percoll (1.060 g/ml) to 80% Percoll (1.096 g/ml) as described before50,87. The various RBC fractions were isolated and washed three times with Ringer’s solution87 by repeated centrifugation for 5 min at 400g before analysis.

Deformability, osmotic fragility and aggregation

Deformability and aggregation were measured using a laser-assisted optical rotational cell analyser (Lorrca MaxSis, Mechatronics, The Zwaag, The Netherlands) as described previously108. Osmotic gradient ektacytometry, i.e. RBC deformability during exposure to a gradient of increasing osmolality, was performed using the osmoscan module of the Lorrca ektacytometer, as previously described108. Osmotic fragility was also assessed by measuring the free hemoglobin concentration at 415
Flow cytometry

The percentage of phosphatidylserine (PS)-exposing RBCs was determined using Annexin V, essentially as described before. One million RBCs were incubated with phycoerythrin (PE)-labeled Annexin V (1:25, BD Pharmingen, Hoeven, the Netherlands) for 20 min at room temperature in the dark. Tert-butylhydroperoxide-treated RBCs (1 mM, 45 min at room temperature) served as positive controls. APC-labeled anti-C3d (1 μg/million cells, Assay Pro, St. Louis, Missouri, USA) was used to evaluate the degree of complement-induced opsonization. APC-labeled CD71 (clone CY1G4, 1:200, Biolegend, San Diego, California, USA) was used to measure the percentage of reticulocytes. Staining of band 3 with eosin-5’ maleimide (EMA, Thermo Fisher Scientific, Landsmeer, the Netherlands) was performed by incubating one million RBCs with 25 μl of EMA (0.5 mg/ml in Ringer’s solution) in the dark at RT for 15 min.

After staining, RBCs were washed three times with Ringer’s solution, and analysed by flow cytometry (FACSCalibur instrument, BD Biosciences, Franklin Lakes NJ, USA) using CELLQuest software (BD Biosciences). Data were analysed with FlowJo cell analysis software v.10 (FlowJo, LLC, Ashland OR) using 200,000 events.

Isolation and characterization of microvesicles

Microparticles (MPs) were isolated from the platelet-rich plasma (PRP) obtained after differential centrifugation as described before. Microvesicle analysis was performed using mixtures of FITC-labeled CD235a (1:100) and PE/Cy5-labeled CD41 (1:10) by flow cytometry as previously described. Sulfate latex microspheres (0.9 μm; Invitrogen, Carlsbad CA, USA), and washed Flow-Count calibration beads (Beckman Coulter, Brea CA, USA) were used for quantification.
Statistical analysis

Differences between donors were determined using paired, repeated measures ANOVA followed by Bonferroni (parametric distribution) or ANOVA Friedman test followed by Dunn’s (non-parametric distribution) correction for multiple comparisons. Two-sided $p$-values less than 0.05 were considered statistically significant.
RESULTS

Percoll gradient-based separation according to density is a commonly used method to isolate RBCs of various ages. This method is based on the observation that, in healthy individuals, density increases with cell age, due to the combined loss of volume, surface area, hemoglobin and water. The loss of water exceeds the loss of hemoglobin, which is the main cause of the increase in density.220 Changes in the RBC pattern after density-based separation are associated with alterations in RBC homeostasis.288,289 After the first day of marching, the RBC distribution over the Percoll layers in most individuals showed a shift towards denser RBCs, followed by a shift towards less dense cells during the second day. These changes were already visible after the first day and in the morning of the second day (Figure 6.1).

![Figure 6.1: Percoll gradients of red blood cells during the first two days of the 2018 edition of the Nijmegen Four Days Marches.](image)

Blood was taken at baseline (day 0-e), in the evening of day 1 (day 1-e), in the morning before day 2 (day 2-m), and after day 2 (day 2-e). After removal of plasma, platelets and white blood cells, RBCs were separated according to density using Percoll gradients, as described in Materials and Methods. The figure shows the representative results of one donor: a shift towards denser RBCs after the first day of marching (arrows) and a shift towards lighter RBCs during the second day (arrow head). Similar patterns were seen in all donors.
Participation in the Four Days Marches led to an overall steady increase in RBC volume, as indicated by the increase in the mean value of the forward scatter, and to a concomitant decrease in the mean fluorescence intensity of the membrane protein probe EMA (Figure 6.2).

![Figure 6.2: Changes in forward scatter and EMA binding of red blood cells during the Nijmegen Four Days Marches.](image)

A, mean forward scatter (FSC) extracted from the flow cytometry data; B, the mean fluorescent intensity (MFI) after binding of EMA, as described in Materials and Methods. Each symbol represents one donor. Significant differences: *, p<0.05; **, p<0.01; *** p<0.001; ****, p<0.0001 (N=10).

In all subjects, hemolysis slightly increased during the marching days from 0.25 ± 0.03 (N=15) to 0.39 ± 0.14 (N=14) absorption units. The increase in intravascular hemolysis was accompanied by a decrease in the fraction of phosphatidylserine-exposing RBCs after each day of marching (Figure 6.3A). Also, the fraction of C3d-positive RBCs increased significantly in the morning before the second day of marching (Figure 6.3B, Day 2-m). The complement-containing and PS-positive RBCs are likely to be the oldest RBCs that are most susceptible to exercise-induced changes in membrane organization and/or composition.\(^{151,244}\)
How RBC homeostasis is affected by exercise

Figure 6.3: Appearance of removal signals on red blood cells during the Nijmegen Four Days Marches.
A, the fraction of phosphatidylserine (PS)-exposing RBCs as determined using fluorescent annexin V; B, the fraction of C3d-positive RBCs, as determined with a fluorescent monoclonal antibody, as described in Materials and Methods. Each symbol represents an individual. Significant differences: *, p<0.05; **, p<0.01; *** p<0.001; ****, p<0.0001 (N=10).

The fraction of the most mature reticulocytes, as detected by the fraction of CD71-positive RBCs, decreased after the first day of marching, but increased again during the consecutive night (Figure 6.4). These analyses gave no indications for disturbed reticulocyte maturation.

Figure 6.4: Changes in the fraction of reticulocytes during the Nijmegen Four Days Marches.
A, the numbers of reticulocytes were determined using fluorescent anti-CD71 antibody in combination with thiazole orange: R1, CD71 negative, R2, low CD71 expression, R3, medium CD71 expression, R4, high CD71 expression.
high CD71 expression; B, percentage of each reticulocyte maturation fraction group per day. Significant differences: *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001 (N=10).

Physiological RBC aging, but also RBC-centered pathologies and semi-physiological stress in vitro are accompanied by the generation of microvesicles. In the blood of the Four Days Marches participants, we found no significant changes in RBC-derived microvesicle concentrations. The concentrations of platelet-derived microvesicles, however, increased significantly after each marching day (Figure 6.5).

![Figure 6.5: RBC-derived and platelet-derived microvesicles in the blood of participants in the Nijmegen Four Days Marches. A, concentration of RBC-derived microvesicles; B, concentration of platelet-derived microvesicles. Microvesicles were isolated from the blood of Four Days Marches participants and their concentrations were measured using RBC-specific and platelet-specific markers as described in Materials and Methods. Post-test significant differences: *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001 (N=10).](image)

Aging-associated and stress-induced changes in membrane organization do not only affect RBC integrity, recognition and removal, but also their behavior in the circulation. Aggregation is a key characteristic of RBCs, as it reflects their interaction with each other and with plasma proteins, and affects hemostasis and thrombosis. Participation in the Four Days Marches induced a decrease in RBC aggregation after the first day, and an increase in deformability after two days (Figure 6.6).
Fig. 6.6: Red blood cell deformability and aggregation during the Nijmegen Four Days Marches.

The tendency to aggregate (Aggregation Index, Al in panel A), maximal deformability (Elongation Index, El in panel B), and the elongation kinetics during increased stress (SS ½ in panel C) were measured using an LORRCA ektacytometer as described in Materials and Methods. Post-test significant differences: *, p<0.05; **, p<0.01; *** p<0.001; ****, p<0.0001 (N=10).
DISCUSSION

Our data indicate that physiological stress induced by prolonged exercise as experienced by participants in the Nijmegen Four Days Marches induces an early response in the RBC compartment of the circulation. Earlier research has shown that this may be considered a moderate-intensity exercise of approximately 70 percent of the maximal heart rate. The changes in cell volume and density (Figure 6.1 and 6.2) may, by themselves, not have a physiological effect, but they indicate a shift towards an overall younger RBC population, mainly by an early disappearance of the oldest RBCs. This may be partly due to mechanical stress-induced breakdown, as suggested by the slight increase in intravascular hemolysis, in line with a previously reported decrease in haptoglobin and increase in ferritin after the first march day. Hemolysis occurs in all types of exercise, presumably resulting from mechanical damage when RBCs pass through the capillaries of the foot, or as the result of increased circulation and/or large muscle contractions. On the other hand, an exercise-associated increase in blood flow may stimulate exosome-mediated removal of RNA and fragments of cellular organelles, concomitant with a rearrangement of the cell membrane, and may thereby accelerate the maturation of reticulocytes (Figure 6.4). Such a maturation-catalyzing effect of mechanical stimulation may be involved in the final differentiation of RBCs cultured in vitro.

The appearance of phosphatidylserine in the outer leaflet of the RBC membrane and the accumulation of activated complement (Figure 6.3) may contribute to increased RBC phagocytosis. These RBCs are likely to be the oldest RBCs in the population, since they are the most susceptible to hyperosmotic conditions in vitro. The same stress mechanism may be operative in vivo, in view of the light hypernatremia due to inadequate fluid intake in the first days of the Four Days Marches. In addition, exercise may be accompanied by increased oxidation and methemoglobin formation, especially in the oldest RBCs.

The hypothesis that a shift to an overall younger RBC population results in a functional improvement of the RBC compartment is supported by our observation that RBC deformability increased, whereas aggregation decreased (Figure 6.6), both improving blood flow and oxygen delivery. A decrease in the tendency of the RBCs to form aggregates has been postulated to be associated with the loss of old RBCs with less sialic acid on their membrane, resulting in a higher average cell
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surface charge of the resulting RBCs\textsuperscript{13,293}. Shifts in deformability and aggregability with a concomitant increase in function have been reported for various forms of exercise, varying from the effects of dancing on deformability and aggregation in elderly women\textsuperscript{294,295} to the positive correlation between RBC deformability and maximum running velocity and VO2 peak in well-trained athletes\textsuperscript{283,296,297}.

\textit{In vitro}, semi-physiological stressors induce RBC vesiculation\textsuperscript{98,165}, but we did not observe increased RBC-derived microvesicle concentrations in the blood of our participants (Figure 6.5). This is probably due to the fast removal of RBC-derived microvesicles by the reticulo-endothelial system\textsuperscript{161}. However, we observed a significant increase in the concentration of platelet-derived vesicles (Figure 6.5), supporting previous conclusions that exercise may activate platelets and induce platelet precursor mobilization\textsuperscript{298–301}. These processes may be increased by exercise-induced inflammatory responses as observed during running\textsuperscript{281}, but also during the first day of the Four Days Marches\textsuperscript{287}. Similarly, inflammation has a pronounced effect on RBC membrane organisation and stability\textsuperscript{98,165}, to the extent of causing anemia\textsuperscript{302}.

Thus, our data support the hypothesis that exercise causes mechanical stress and inflammation that are likely to induce removal of the oldest, most susceptible RBCs. This occurs already during the first day of moderate-level exercise, and represents an early homeostatic adaptation resulting in a functionally improved RBC population. Our data also support current theories on the cellular mechanisms of physiological aging and removal of RBCs \textit{in vivo}, which have been mostly deduced from observations and interventions \textit{in vitro}.

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\section*{Conflict of Interest}

The authors declare no conflict of interest.
Red blood cell stress: a challenge to homeostasis
GENERAL DISCUSSION

The journey that a red blood cell (RBC) has to take during its lifespan comes with many struggles. In order to be able to execute its function effectively, the RBC has various mechanisms and structures to handle intracellular and extracellular stressors. Nevertheless, lesions that impact cell structure and function occur at different levels, depending on stress intensity and duration, both in physiological and non-physiological conditions. The subsequent adaptations may impair RBC homeostasis, as they may lead to changes in deformability, exposure of removal signals and, ultimately, disappearance from the circulation.

Vesiculation, an adaptation process

Vesiculation is a consequence of stress and constitutes an integral part of RBC homeostasis. During their lifespan in the circulation, RBC volume, hemoglobin content, and membrane content and composition and surface decrease by 30%, 20%, and 20%, respectively, mainly due to vesiculation. This process not only plays a role in all stages of the RBC life and in RBC pathophysiology, but also induces irreversible storage lesions. Hence, the concentration and composition of vesicles constitute biomarkers for RBC homeostasis. We reviewed the currently available knowledge of the composition and production of RBC-derived microvesicles in Chapter 2, and concluded that RBC-derived microvesicles are produced in response to a variety of physiological and non-physiological triggers, and that their composition reflects the stress that cells experience. However, the processes that lead to vesicle production under different conditions are yet to be discovered, whereas the limitations of the current technology to isolate and characterize vesicles, present major challenges to this field of research.

Unveiling the complete composition of the RBC-derived vesicles is a challenge especially because of their size (approximately 200-300 nm), which impedes isolation and detection. In general, differential centrifugation remains the most regular method for vesicles isolation, although other techniques have been applied to avoid centrifugation limitations such as the difficulty to process large volumes and the possibility of vesicle damage. For instance, size exclusion chromatography and immune affinity are techniques that reduce these restrictions, but they still bring
others, e.g. contamination with (lipo)proteins and of the effect of the affinity reagents and epitope/ligand density on different vesicle types, respectively\textsuperscript{304,305}.

Flow cytometry, immunoblotting, ELISA, dynamic light scattering (DLS), electron microscopy (EM), cryo-EM, and -Omics techniques can all aid in the detection and characterization of microvesicles, but the analytical struggles to identify these small particles persist and the lack of a gold standard for isolation results in poor interlaboratory reproducibility.

During storage, vesiculation leads to the appearance of spherocytes, and the associated decrease in deformability may thereby contribute to the fast removal of 25\% of transfusion RBCs within the first hours after transfusion\textsuperscript{9}. Also, antigens/signals on vesicles may trigger immunological and inflammatory transfusion reactions, especially in transfusion-dependent patients. Chapters 3 and 4 show the changes in number and composition of vesicles during RBC storage in transfusion units in the blood bank.

In Chapter 3 we show that the fraction of inactivated AChE is increases in vesicles that accumulate during storage. Vesicles shed during storage time are enriched in this GPI-linked protein as already described before\textsuperscript{241,306}. Together with our findings this confirms previous proteomic and immunological data showing that damaged membrane components are removed by vesiculation, both \textit{in vivo} and \textit{in vitro}\textsuperscript{190,240}.

Chapter 4 reinforces the effect of storage lesions and the time of circulation on vesiculation, by showing that during circulation in an extracorporeal device, the numbers of microvesicles produced by stored RBCs increased, not only depending on circulation but also on storage time. During surgery, the microvesicle numbers decreased after the first hour of circulation/surgery time, probably due to rapid immune recognition and removal. Methemoglobin increased with storage time and circulation time, as seen before\textsuperscript{233}, and its concentration was positively correlated with vesicle numbers, indicating that oxidative stress and hemoglobin damage may be central processes in the vesiculation mechanism.

As approximately 90 million RBC units are transfused worldwide each year, the results of these studies highlight the demand for more research on the transfusion impact of RBC-derived microvesicles and their adverse clinical effects. Investigation of RBC-derived vesicles would lead to a better understanding of pathophysiological
conditions such as inflammation and thrombosis, since the vesicles carry antigens and pro-coagulation factors that enhance those pathways, and the vesiculation process leads to irreversible changes in cell rheology and morphology that may contribute to thrombosis as well. Moreover, essential features of vesicles, not investigated in depth in this thesis but that definitely remain to be addressed in the future, are the thrombogenic activity of RBC-derived and platelet-derived vesicles that are shed under stress. Furthermore, approaches such as proteomics, lipidomics and metabolomics may provide insight into vesicle generation during storage and the translation of this knowledge into an understanding of the related pathophysiology in vivo.

The discrepancies between physiological and non-physiological environments play a role in microvesicle homeostasis and have an impact on their detection. Microvesicles are present in a concentration of approximately 1000 microvesicles/L in plasma, but in vitro the concentration increases exponentially with storage time. Microvesicles generated in vitro do not have to deal with the reticuloendothelial system, that removes vesicles very rapidly. The effectiveness of the reticuloendothelial system restricts the potential harmful thrombotic and inflammation effects. This system may become overloaded on transfusion settings when high volumes of stored RBCs and vesicles are infused into the patient.

The investigation of RBC-derived microvesicles in vivo in the blood of patients with PNH, described in Chapter 5, generated new perspectives on the causes and consequences of microvesicle generation. Remarkably, the formation of microvesicles by RBCs is altered in patients with PNH, as indicated by the considerable numbers of PS-negative (no Annexin V reactivity), low CD59 (lower anti-CD59 MFI in comparison to control samples) and CD59 negative (no anti-CD59 reactivity) microvesicles. Interestingly, these microvesicles are also present in the blood of control donors, albeit in lower amounts. This finding implicates different mechanisms of vesicle production and/or suggest conformational changes in these antigens, maybe due to ageing related stress, that may prevent antibody binding and thus detection.

It has been documented that not all microvesicles expose PS, which suggests the presence of different populations of microvesicles. These may carry a variety of phospholipids and proteins, such as CD55 and CD59, on their surfaces mirroring
the diversity of mechanisms of microvesicle production, not only in RBCs but in platelets as well. PS exposure by itself does not necessarily lead to vesiculation in platelets, and some microvesicles shed by unstimulated platelets expose PS\textsuperscript{311}. Moreover, Annexin V, the widely used probe for vesicle analysis, is present in the blood, which could shield exposed PS. Also, conformational changes in membrane organization or calcium might also lead to false negative results. The use of another marker with different binding characteristics such as lactadherin\textsuperscript{312}, may overcome these limitations.

The microvesicles that are shed by RBCs are enriched in the cellular complement regulators CR1, CD55, CD59\textsuperscript{313}. Therefore, RBCs may no longer be protected from opsonization and complement-induced lysis, which enhances their disappearance from the circulation. In addition, enriched CR1 expression in microvesicles may increase the concentration of immune complexes. This does not have a direct effect on RBCs, but contributes to the progression of disease by the deposition of these immune complexes in tissues and the induction of an inflammatory response.

Vesiculation has been postulated to contribute to protection against complement-induced cell death by the selective elimination of the membrane attack complex in a calcium-dependent manner\textsuperscript{313,314}. High numbers of circulating vesicles are present in patients with inflammatory disorders\textsuperscript{98}, which is associated with enhanced complement activation\textsuperscript{315}. Thus, RBCs might balance overwhelming complement activation by shedding microvesicles and, furthermore, microvesicles might act as complement scavengers by carrying opsonins and sacrificing themselves via MAC destruction\textsuperscript{313,314}. Also, PNH cells have a higher oxidative status when compared to healthy cells\textsuperscript{316}. It is not clear if this is a result of free hemoglobin, platelet hyperactivity or a pre-existing defect in PNH cells that exacerbates oxidative stress. Increased oxidation may affect vesiculation by damaging band 3, hemoglobin and cytoskeleton components, formation of oxidized phospholipids and proteins rafts, all leading to an abnormal composition of RBC-derived microvesicles.

In addition, microvesicle-mediated transfer of CD55 and CD59 from healthy RBCs to RBCs without these proteins and vice-versa has been reported\textsuperscript{205}, which may have positive as well as deleterious effects on RBC homeostasis. Microvesicle-RBC fusion is more likely to occur \textit{in vitro} in the blood bank, due to the high concentration of vesicles in RBC concentrates and the absence of the
reticuloendothelial system. Such exchange of molecules may open ways for the development of PNH treatment tools, e.g. by using healthy vesicles to transport GPI-linked proteins to PNH affected RBC in vitro, and for an increase in the understanding of the mechanisms involved in communication between RBCs.

RBC-derived microvesicles are not the most common microvesicle phenotype in plasma, this title is held by platelet-derived microvesicles. Such differences not only are related to their biological differences (activation, numbers in circulation), but also may be caused by variances in blood sampling and handling procedures (e.g. needle Gauge, use of tourniquet, anticoagulant choice, buffer, centrifugation steps, time and speed, freezing and thawing).

In the blood of patients with PNH, the number of platelet-derived microvesicles is higher than in the blood of control donors, which is likely the effect of oxidative stress and complement attack that activate platelets rather than destroy them. The majority of the platelet-derived microvesicles is CD59-negative. These data strongly suggest that the absence of GPI-linked proteins stimulates microvesicle production by platelets. The lack of GPI-linked proteins also affects the microvesicle composition, as suggested by the increased numbers of PS-negative platelet-derived microvesicles in the blood of PNH patients in comparison to control donors. The PS-negative microvesicles may survive longer in the circulation, which may augment their putative contribution to the thrombotic status of patients with PNH.

Platelet-derived vesicles together with the other PNH-associated pro-thrombotic factors such as RBC-derived vesicles, NO depletion and free hemoglobin contribute to thromboembolism, the most common cause of mortality in patients with PNH. Our data enable the application of more targeted therapies to diminish disease burden (e.g. anticoagulant treatment and thrombolysis management together with the use of other complement inhibitor-based treatment specially in countries where eculizumab is not available) and improve patient outcome in the near future. Microvesicles carry a high density of phospholipids and receptors for coagulation factor Va and VIII, leading to approximately 50- to 100-fold higher procoagulant properties than an identical surface area unit of an activated platelet. Microparticles may have hemostatic as well as pathological roles in thrombus formation. It is very likely that they play an important role in the prothrombotic setting of PNH as they do in trauma, sepsis, and cancer. Moreover, vesicles of...
PNH patients may enhance endothelial activation and vascular inflammation\(^{320}\), another contribution to thrombosis.

Vesicle generation by platelets was also induced by exercise during the Four Days Marches, as described in Chapter 6. This may be caused by exercise-induced inflammatory, oxidative, and mechanical stress\(^{311}\). Alterations in circulating microvesicle concentrations with exercise seem like a normal response in healthy adults, but little is known about the influence of acute and chronic exercise upon the cargo and characteristics of microvesicles and their dynamics in specific populations. Monitoring changes in circulating microvesicle concentrations prior to and after exercise in healthy controls and patient populations (i.e. cardiovascular patients) could provide further insight into microvesicle dynamics and could result in the use of exercise-derived microvesicles as biomarkers in clinical diagnosis. Several studies have already stated associations of microvesicle (including platelets and RBC-derived ones) concentrations and contents with cardiovascular diseases, for instance\(^{321}\).

Interestingly, besides the already discussed deleterious effects, there is evidence linking platelet microvesicles to adhesion and differentiation of endothelial cells\(^{322}\). This suggests that exercise-induced microvesicle release may improve vascular repair and function by enhancing differentiation of circulating endothelial progenitor cells. The equilibrium between injury and repair may rely on the concentration and composition of these vesicles, and the biological history of the individual. Integration of in vitro and in vivo studies exploring these ideas may help unravel the mechanisms of exercise-mediated vascular adaptations. These studies will profit from research exploring the detailed dynamics of microvesicles in reaction to specific exercise variables, and may unveil their physiological role.
RBC challenges under stress

In vitro:

Stress occurs not only during physiological aging in vivo, but also during storage in the blood bank, and in various clinical circumstances. The data presented in Chapter 3 and Chapter 4 support the hypothesis that mechanical stress and - not yet identified - stressors of blood bank storage are likely to affect especially the oldest RBCs, inducing their removal from the circulation upon transfusion.

Analysis of aging of RBCs in blood bank conditions, as described in Chapter 3, shows that neither AChE expression nor enzymatic activity decreased significantly with storage time, confirming previous data. These findings, compared to what occurs during aging in vivo and especially in 1-day stored RBCs that show steady decrease in enzyme activity with ageing and storage process, respectively, emphasize that different environments may contain different stressors with different effects on RBC aging. The enrichment of AChE in storage-derived vesicles and the decrease in AChE activity with aging suggest that damaged AChE molecules are shed in microvesicles. The preferential location of GPI-linked proteins in rafts makes it likely that a raft-based process is part of the vesiculation mechanism.

Moreover, CD59 concentrations did not change during storage time. Other studies reported similar findings, whereas some studies showed a decrease of CD59. Such incongruity could be attributed to differences in blood bank methodology, storage buffer composition, leukodepletion, and the strategies applied during RBC analysis.

Storage lesions may become harmful after transfusion, which is especially relevant for patients undergoing extracorporeal circulation-assisted surgery. The contact with artificial surfaces induces changes in RBCs, especially by affecting bending properties and membrane tension. Therefore, the general purpose of Chapter 4 was to determine the effect of circulation in a heart-lung machine on RBC homeostasis. We compared the data obtained from a stand-alone heart-lung machine approach with those obtained during EC-assisted cardiac surgery, in order to be able to differentiate between EC-induced alterations and patient-associated adaptations. Our results show an increased susceptibility to osmotic stress, impaired deformability, increase in aggregation and the appearance of removal
signals during circulation of RBCs in a heart-lung machine, both in stand-alone settings and during surgery. A considerable number of the patients that undergo EC-assisted cardiac surgery receive RBC transfusion before the surgery, and the stressors that are active during isolation and storage will be transfused along with the cells, for instance, vesicles which can be depleted using prior filtration of blood products what may yield substantial improvement of transfusion safety. Our observations suggest that the extent and kinetics of the RBC alterations depend on the natural history of the RBCs, in particular the length of storage time in the blood bank. This is in agreement with previous data showing that transfusion of RBCs that had been stored for more than two weeks in children up to 18 years old undergoing cardiac surgery highly increases the risk of postoperative complications, such as infection and inflammation, and reduces short- and long-term patient survival. Furthermore, post-pump chorea has been described in children that have undergone EC-assisted cardiac surgery. Brain damage and oxidative stress have been reported in these patients, and deep hypothermia and circulatory arrest are frequently linked to it. It has been postulated that altered RBC rheology may be involved in the underlying neuropathophysiology. Associations between storage time and post-surgical complications were not found in adult patients, implying the involvement of an immature immune system in childhood post-pump chorea. Follow-up studies with children that were under EC may be a fruitful source of further and deeper insights in this area.

Chapter 4 presents biophysical, biochemical and immunological data that can be applied for the improvement of EC and other flow-assist device technology. The significance of these data will be enhanced by increasing the number of observations/patients, and by including patients undergoing longer EC/surgery time and/or more transfusions. Our data also provide a background for comparing various pumps in use, and their impact on hemolysis and RBC-mediated inflammatory responses, since mechanical stress and its extent is expected to have a direct impact on these parameters. Such studies and evaluations are in view of the fast pacing evolution in EC technology.
**In vivo:**

Physiological cell aging impacts RBC membrane structure and functional signaling. In Chapter 3 we observed an age-dependent decrease in the enzymatic activity of the GPI-anchored protein AChE during RBC aging in vivo. This decrease, which starts early in RBC life, may constitute a sensitive marker of aging in vivo and is associated with band 3 phosphorylation status, linking it to changes in membrane organization. However, we found no aging-associated decrease in the amount of this protein, similar to the constant number of other GPI-anchored proteins such as CD55 and CD59. These data suggest that stressors involved in aging in vivo may cause only minor changes in membrane protein content, but major changes in protein conformation and consequently in protein function. Also, the decrease in concentration of these molecules on the RBC surface as a result of vesiculation is likely to be masked by the fact that RBCs are analysed mostly as a total population, without taking into account the variability in the activity of aging-associated and/or vesiculation-affecting processes.

The capacity to block complement attack and opsonization is a major determinant of RBC aging-related homeostasis and removal. Also, the (lack of) functional activity of AChE, CD55, and CD59 on vesicles emphasizes the role of vesiculation in the removal of damaged, non-functional molecules. Thus, more (detailed) research on the functional parameters of GPI-linked proteins is needed to understand the aging process that red blood cells undergo in vivo.

The clinical heterogeneity of the patient population and the relatively large variability in RBC homeostasis parameters in control donors hampered robust conclusions, but our data did not indicate an abnormal RBC aging process in patients with PNH. However, the majority of the RBCs contained some CD59 and CD55. Since mature RBC do not have a protein synthesizing machinery, genome editing tools applied to erythropoiesis in vitro (e.g. CRISPR techniques applied to gene therapy and targeted gene mutation) can be used to better understand the role of GPI-linked proteins in RBC differentiation and aging-associated removal.

The RBC unceasingly undergoes mechanical and oxidative insults during its 120 days lifespan in the bloodstream. Mechanical stress does not overtly damage the RBC under physiological conditions but, in combination with oxidative stress, it may induce a variety of changes ranging from decreased deformability to outright lysis.
Oxidative stress may be particularly active in the microcirculation, where the RBCs are in close contact with the vasculature and take up exogenous ROS\textsuperscript{291}. Together with the endogenous ROS, resulting from autoxidation of Hb, and the gradual decrease in antioxidant protection by catalase, GSH, and PRDX-2\textsuperscript{340,341}, oxidation-induced changes in RBC components impair RBC function in a time-dependent, aging-associated manner\textsuperscript{13,50}.

During exercise, increase in circulation rate, elevated body temperature, musculature compression, elevated catecholamine concentrations, dehydration, acidosis and elevated ROS formation in the plasma all have an impact on RBC homeostasis\textsuperscript{303}. Exercise leads to hemolysis by an oxidation-mediated mechanism in sedentary, but not in trained individuals\textsuperscript{13,342–344}. Exercise-associated increases in RBC deformability in trained individuals have been postulated to reflect a shift in the RBC population, due to increased removal of the old RBCs and a predominance of the younger RBCs with a higher deformability\textsuperscript{303}. This phenomenon was also noticed in patients undergoing EC-assisted cardiac surgery as shown in Chapter 4.

High oxidative stress affects the structure of RBCs and reduces their capacity to deform and pass through small capillaries\textsuperscript{13,50}. An increase in RBC membrane rigidity is a result of lipid oxidation after a single bout of maximal and submaximal exercise\textsuperscript{124}. Furthermore, senescent RBCs are more sensitive to oxidative damage than the young and middle-aged populations\textsuperscript{13}.

The data presented in Chapter 6 indicate that moderate-level exercise by participants of the Nijmegen Four Days Marches indeed induces fast removal of the oldest RBCs providing a cellular, homeostatic mechanism for early adaptation by leading to a younger, functionally improved RBC population\textsuperscript{342,344}. In addition, these data indicate that longitudinal studies of the effects of exercise on RBC aging-associated characteristics will enable mechanism-based investigations in related research areas, such as the effect of targeted antioxidants in RBC redox biology\textsuperscript{119,120,345}.

In addition, studies using various exercise modalities and intensity, and also a variety of populations, which might have a significantly diverse impact on pro- and antioxidant stimulation, RBC adaptive or regenerative properties and senescence are interesting subjects of investigation in the field of RBC homeostasis-associated sport physiology.
CONCLUSION

RBC biology deserves extensive research, since many internal and environmental changes affect RBC survival and function. In this thesis we investigated how RBC homeostasis is affected by various stress conditions in different environments. We have acquired more insight into the mechanisms underlying RBC homeostasis in vivo, in vitro, and ex vivo, i.e. in a combination between in vivo and in vitro conditions. Our results may be helpful especially in understanding pathophysiological and iatrogenic stress and its impact on (the assessment of) clinical outcomes. Moreover, this knowledge may assist the development of mechanism-based interventions.
General Summary
Algemene Samenvatting
Resumo Geral
Chapter 8

Summary
GENERAL SUMMARY

Any disturbance of the equilibrium between production of young and removal of old RBCs by endogenous and exogenous stressors has various cellular and systemic consequences. In this thesis, we investigate how RBC homeostasis is affected by various stress conditions in different environments and pathologies. We have acquired more insight into the mechanisms underlying RBC homeostasis in vivo, in vitro, and ex vivo. Our results contribute to the understanding of how pathophysiological and iatrogenic stress affect (the assessment of) clinical outcomes. This knowledge may contribute to the development of mechanism-based interventions.

Chapter 1 presents a general introduction to RBC homeostasis and the stress that RBCs undergo in physiological as well as non-physiological conditions. We highlight the stressors that have an impact on metabolism, membrane structure, and function, as well as the corresponding adaptations to avoid premature removal.

The review presented in Chapter 2 aimed to reach a better understanding of the key mechanisms underlying microvesicle generation in several conditions, and the role of RBC-derived microvesicles in pathophysiology. The thus obtained knowledge showed that RBC-derived microvesicles may be actively involved in several pathophysiological processes and that this may become of clinical importance, especially in transfusion settings. Furthermore, these insights gave rise to the hypotheses and interpretations of the following chapters that investigate RBC homeostasis under several environmental stress conditions.

There is no unambiguous biomarker for RBC ageing in vivo or in vitro for use in RBC homeostasis studies, but determination of AChE characteristics may yield information on the mechanism of ageing-associated vesicle formation and lead to the establishment of an RBC-aging marker. In Chapter 3, we investigated whether there were any changes in AChE during aging in vivo and in vitro. The data showed that AChE activity, but not AChE amount may constitute a sensitive biomarker of RBC ageing in vivo, but not in vitro. This finding highlights the differences between physiological and non-physiological conditions. Also, the presence of non-functional AChE confirms the role of microvesicles in the removal of damaged proteins.
Chapter 4 reports the impact of extracorporeal circulation during heart surgery on in RBC homeostasis. We demonstrate that circulation in a heart-lung machine is accompanied by changes in RBC volume, an increase in osmotic fragility, changes in deformability and aggregation behavior, alterations in exposure of phosphatidylserine and in microvesicle generation. These changes are key characteristics of the RBC aging process, and thus likely increase RBC susceptibility to the various mechanical, osmotic and immunological stress conditions encountered during and after surgery in the patient’s circulation, thereby contributing to the side effects of surgery. Moreover, these aging-related parameters provide a foundation for validation and improvement of extracorporeal circulation technology in vivo as well as in vitro.

In vivo, pathophysiological conditions may disrupt the homeostatic balance of the response to cellular stressors. For example, RBCs from patients with Paroxysmal Nocturnal Hemoglobinuria (PNH) are deficient in GPI-linked proteins, due to a mutation in the PIG-A gene. The consequent deficit in complement regulators CD55 and CD59 causes RBC stress consisting of complement deposition, MAC formation and ultimately hemolysis89. Triggered by these observations, we postulated that the presence of intact GPI-anchored proteins is essential for membrane organization and thereby for RBC morphology, function, and survival in vivo. In Chapter 5, we present the results of an exploratory examination of various structural and functional parameters of RBCs from patients with PNH. Although no major aberration of the physiological RBC aging process in patients with PNH was discovered, our results on the characteristics of RBC-derived microvesicles in PNH patients indicate that microvesicle generation may affect RBC lifespan and affect hemostasis and thrombosis.

The effect of exercise on RBC homeostasis is described in Chapter 6, where we report on an exercise-induced shift in RBC age to a younger and functional improved population. Our data on hematological, functional and structural characteristics of RBC aging under physiological stress in vivo during exercise, supports the hypothesis that exercise causes mechanical stress and inflammation that are likely to induce removal of the oldest, most susceptible RBCs303. This process constitutes an early homeostatic adaptation to stress. Moreover, these data obtained in vivo enrich the RBC homeostasis research field on ageing mechanisms, which relies mostly on conclusions that are drawn from studies performed in vitro.
Elke verstoring van het evenwicht tussen de aanmaak van jonge, en de verwijdering van oude rode bloedcellen (RBC) als gevolg van endogene en exogene stressoren heeft cellulaire en systemische gevolgen. In dit proefschrift onderzoeken we hoe de homeostase van RBC beïnvloed wordt door verschillende stressoren, in verschillende omgevingen en bij verschillende ziektebeelden. Dit heeft tot meer inzicht geleid in de mechanismen die de homeostase regelen \textit{in vivo}, \textit{in vitro} en \textit{ex vivo}. Onze resultaten dragen daardoor bij aan een beter begrip van het effect van pathofysiologische en iatrogene stress op (de interpretatie van) klinische bevindingen. Deze kennis is ook van belang voor de ontwikkeling van interventies gebaseerd op moleculaire en cellulaire mechanismen.

\textbf{Hoofdstuk 1} is een inleiding in de homeostase van RBC en de stress die RBC ondervinden in fysiologische en pathologische omstandigheden. We besteden vooral aandacht aan stressoren die een impact hebben op het metabolisme, de structuur van de celmembraan en functie van RBC, en aan de aanpassingen die RBC doen om voortijdige verwijdering te voorkómen.

\textbf{Hoofdstuk 2} geeft een overzicht van de belangrijkste mechanismen die ten grondslag liggen aan de productie van microvesikels onder verschillende omstandigheden, en van de biologische activiteit van deze microvesikels. Dit overzicht laat zien dat microvesikels die afkomstig zijn van RBC, betrokken zijn bij verschillende pathofysiologische processen en dat dit van klinisch belang is bij bloedtransfusies. Dit overzicht inspireerde ons ook tot de hypotheses en interpretaties van het onderzoek naar de homeostase van RBC under verschillende stressvolle omstandigheden.

Er bestaat geen eenduidige ‘biomarker’ voor de leeftijd van RBC die bruikbaar is bij het in kaart brengen van de homeostase van RBC. Bepaling van de eigenschappen van het enzym acetylcholinesterase (AChE) leek informatie te kunnen opleveren over het mechanisme van de verouderingsgerelateerde vorming van microvesikels, én te kunnen leiden tot een geschikte biomarker voor de leeftijd van RBC. Dit was het onderwerp van het in \textbf{Hoofdstuk 3} beschreven onderzoek. De resultaten van dit onderzoek laten zien dat de activiteit van AChE, maar niet de hoeveelheid, een gevoelige biomarker is van veroudering \textit{in vivo}, maar niet van veroudering \textit{in vitro}. Deze conclusie benadrukt de verschillen tussen fysiologische
Algemene Samenvatting

en niet-fysiologische veroudering. Bovendien bevestigen onze resultaten de rol van microvesikels in de verwijdering van tijdens het verouderingsproces beschadigde eiwitten.

**Hoofdstuk 4** beschrijft de impact van extracorporale circulatie van RBC tijdens hartoperaties. Circulatie van RBC in een hartlongmachine gaat gepaard met veranderingen in hun celvolume, een toename in hun osmotische gevoeligheid, veranderingen in vervormbaarheid en aggregatie, in het verschijnen van fosfatidylserine aan de buitenkant van de RBC, en in de vorming van microvesikels. Deze verouderingsgerelateerde veranderingen leiden tot een verhoogde gevoeligheid voor mechanische, osmotische en immunologische stressoren en dragen daardoor bij aan de minder gewenste effecten van een operatie. Bovendien zijn deze parameters geschikt voor het evalueren, valideren en verbeteren van extracorporale circulationsystemen.

*In vivo* kan de homeostatische balans van RBC beïnvloed worden door een pathofysiologische verstoring van de reactie op stressoren. Zo zijn de RBC van patiënten met paroxysmale nocturnale hemoglobinurie (PNH) door een mutatie in het *PIG-A* gen deficiënt in de synthese van GPI-verankerde eiwitten. Het daardoor veroorzaakte defect in de complement-regulerende eiwitten CD55 en CD59 leidt tot stress door depositie van complement en de daaropvolgende vorming van het ‘membrane attack complex’ en hemolyse. Op basis hiervan postuleerden wij dat de aanwezigheid van intacte GPI-verankerde eiwitten essentieel is voor een goed georganiseerde celmembranaan en daardoor voor morfologie, functie en overleving.

*In Hoofdstuk 5* presenteren we de resultaten van een verkennend onderzoek van verschillende structurele en functionele parameters van de RBC van patiënten met PNH. We vonden hierbij geen grote afwijkingen van het normale verouderingsproces van de RBC. Wel wijzen onze resultaten er op dat de vorming van microvesikels gevolgen heeft voor hemostase en trombose.

*In Hoofdstuk 6* laten we zien hoe inspanning een verschuiving veroorzaakt naar een jongere en functioneel verbeterde RBC populatie. We rapporteren een aantal hematologische, functionele en structurele bevindingen over de veroudering van RBC tijdens fysiologische stress. Deze bevindingen steunen de hypothese dat de tijdens inspanning optredende mechanische stress en ontsteking de verwijdering van de oudste, meest kwetsbare RBC induceren. Dit proces vertegenwoordigt een
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snelle homeostatische adaptatie aan fysiologische stress. Bovendien zijn deze resultaten van algemeen belang voor een beter inzicht in de manier waarop homeostase van RBC in vivo functioneert, aangezien het beeld hierover vooral gedomineerd wordt door conclusies die getrokken zijn op basis van data die verkregen zijn uit onderzoek verricht in vitro.
RESUMO GERAL

Qualquer perturbação do equilíbrio entre a produção de jovens e a remoção de eritrócitos senis por estressores endógenos e exógenos tem consequências celulares e sistêmicas. Nesta tese, investigamos como a homeostase dos eritrócitos é afetada por várias condições de estresse em diferentes meios e patologias. Com isso, adquirimos mais conhecimento sobre os mecanismos da homeostase dos eritrócitos em condições in vivo, in vitro e ex vivo. Nossos resultados contribuem para a compreensão de como os estresses fisiopatológico e iatrogênico afetam tanto a avaliação dos quanto os próprios desfechos clínicos. Tal conhecimento pode contribuir para o desenvolvimento de intervenções baseadas em tais mecanismos.

O Capítulo 1 apresenta uma introdução geral à homeostase dos eritrócitos e ao estresse que os eritrócitos sofrem em condições fisiológicas e não fisiológicas. Destacamos os fatores estressantes que têm impacto no metabolismo, estrutura da membrana e função celular, bem como as adaptações correspondentes que evitam a remoção sistêmica prematura.

A revisão apresentada no Capítulo 2 teve como objetivo alcançar uma melhor compreensão dos principais mecanismos envolvidos na geração de microvesículas pelos eritrócitos sob várias condições, e o papel destas na fisiopatologia. Em conclusão, as microvesículas derivadas de eritrócito podem estar ativamente envolvidas em vários processos fisiopatológicos e que isso pode se tornar de importância clínica, especialmente em casos de transfusão sanguínea. Além disso, tais percepções deram origem às hipóteses e interpretações dos capítulos seguintes que investigam a homeostase do eritrócito sob várias condições.

Não existe um biomarcador inequívoco para o envelhecimento do eritrócito in vivo ou in vitro para uso em estudos de sua homeostase, porém a determinação das características enzimáticas da Acetilcolinesterase (AChE) pode fornecer informações valiosas sobre o mecanismo de formação de vesículas associadas ao envelhecimento celular e levar ao estabelecimento desse possível biomarcador. No Capítulo 3, investigamos alterações na AChE durante o envelhecimento eritrocitico in vivo e in vitro. Os dados mostraram que a atividade enzimática da AChE, mas não sua concentração/célula, pode constituir sim um biomarcador sensível de envelhecimento in vivo, mas não in vitro. Este achado destaca as diferenças entre
condições fisiológicas e não fisiológicas. Além disso, a presença de AChE não-funcional confirma o papel das microvesículas na remoção de proteínas danificadas.

O **Capítulo 4** relata o impacto do uso da circulação extracorpórea durante a cirurgia cardíaca na homeostase dos eritrócitos. Nós demonstramos que a circulação em uma máquina de circulação extracorpórea é acompanhada por mudanças no volume celular, aumento na fragilidade osmótica, alterações na deformabilidade e agregabilidade, exposição de fosfatidilserina (PS) e geração de microvesículas. Essas alterações são características-chave do processo de envelhecimento do eritrócito, e, portanto, provavelmente aumentam a suscetibilidade destes às variações mecânicas, osmóticas e imunológicas durante a cirurgia e após a mesma já na circulação do paciente, contribuindo para efeitos colaterais. Além disso, tais parâmetros relacionados ao envelhecimento fornecem uma base para validação e aprimoramento da tecnologia de circulação extracorpórea *in vivo* e *in vitro*.

Condições fisiopatológicas podem perturbar o equilíbrio homeostático capaz de responder a fatores estressantes *in vivo*. Por exemplo, eritrócitos de pacientes com Hemoglobinúria Paroxística Noturna (PNH) são deficientes em proteínas ligadas ao glicosilfosfatidilinositol (GPI-âncora) devido à uma mutação no gene PIG-A. O estresse consequente do déficit dos reguladores do sistema complemento, CD55 e CD59, acarreta a deposição de fragmentos do complemento (opsoninas), formação do complexo de ataque a membrana, e, por fim, hemólise⁸⁹. Provocados por essas observações, postulamos que a presença de proteínas intactas ancoradas ao GPI-âncora é essencial para a organização da membrana e, portanto, para a morfologia, função e sobrevivência do eritrócito *in vivo*. No **Capítulo 5**, apresentamos os resultados de um estudo exploratório de vários parâmetros estruturais e funcionais dos eritrócitos de pacientes com PNH. Embora não tenha sido descoberta nenhuma grande aberração do processo fisiológico de envelhecimento destas células em pacientes com PNH, nossos resultados sobre as características de microvesículas derivadas destes eritrócitos indicam que a geração de microvesículas pode afetar a sobrevida celular e a trombose característica da patofisiologia desta doença.

O efeito do exercício na homeostase dos eritrócitos é descrito no **Capítulo 6**. Nossos dados sobre características hematológicas, funcionais e estruturais do envelhecimento dos eritrócitos sob estresse fisiológico *in vivo* durante o exercício
apoiam a hipótese de que o exercício provocaria estresse mecânico e inflamação que provavelmente induziriam a remoção de células senis e suscetíveis para fora da circulação. Este processo constitui uma adaptação homeostática inicial ao estresse. Além disso, esses dados obtidos in vivo enriquecem o campo de pesquisa da homeostase dos eritrócitos especialmente sobre envelhecimento celular, que se baseiam principalmente em conclusões tiradas de estudos realizados in vitro.
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Chapter 9

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Chapter 10
NOMENCLATURE

2,3-DPG: 2,3-diphosphoglycerate

ABBREVIATIONS

AC: after circulation
AChE: acetylcholinesterase
AI: aggregation index
ANOVA: analysis of variance
APC: allophycocyanin
ATP: adenosine triphosphate
BC: before circulation
BCAM: basal cell adhesion molecule
BFU-e: erythroid burst-forming units
Ca\(^{2+}\): calcium ions
CaCl\(_2\): calcium chloride
CD235a: cluster of differentiation 235a, Glycophorin A
CD41: cluster of differentiation 41, Integrin alpha-IIb
CD47: cluster of differentiation 47, integrin associated protein.
CD55: cluster of differentiation 55, DAF
CD59: cluster of differentiation 59, MIRL
CD71: cluster of differentiation 71, transferrin receptor
CFU-e: colony-forming unit-erythroid
CMO: ‘Commissie Mensgebonden Onderzoek’
CO\(_2\): carbon dioxide
CPB: cardiopulmonary bypass
CR1: complement receptor 1
CR3: complement receptor 3
CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats
cryo-EM: Cryogenic electron microscopy
Appendices – Nomenclature

**Ctrl:** control sample
**DAF:** decay accelerating factor
**DC:** during circulation
**DLS:** dynamic light scattering
**EC:** extracorporeal circulation
**EDTA:** ethylenediaminetetraacetic acid
**El:** elongation index
**ELISA:** enzyme-linked immunosorbent assay
**EM:** electron microscopy
**EMA:** eosin-5’ maleimide
**Emp:** erythroblast macrophage protein
**FITC:** fluorescein isothiocyanate
**GAPDH:** glyceraldehyde 3-phosphate dehydrogenase
**GPI:** glycosylphosphatidylinositol
**Gran CS:** granulocytes clone size
**GSH:** reduced glutathione
**GTP:** guanosine-5’-triphosphate
**Hb:** hemoglobin
**HEPES:** 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
**Ht:** hematocrit
**IgG:** immunoglobulin G
**KCl:** potassium chloride
**LDH:** lactate dehydrogenase
**Leuk:** leukocytes
**Lu:** Lutheran blood group
**MAC:** membrane attack complex
**MCH:** mean corpuscular hemoglobin
**MCHC:** mean corpuscular hemoglobin concentration
**MCV:** mean corpuscular volume
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**MFI:** mean fluorescence intensity

**MgSO₄:** magnesium sulfate

**MIRL:** membrane inhibitor of reactive lysis

**MV:** microvesicle

**NaCl:** sodium chloride

**NADH:** reduced nicotinamide adenine dinucleotide

**NADPH:** reduced nicotinamide adenine dinucleotide phosphate

**NO:** Nitric Oxide

**NT:** patient not being treated

**PC:** phosphatidylcholine

**PE:** phosphatidylethanolamine

**PE:** phycoerythrin

**PIG-A:** phosphatidylinositol N-acetylglucosaminyltransferase subunit A

**Plt:** platelet

**PNH:** paroxysmal nocturnal hemoglobinuria

**PRDX-2:** peroxiredoxin-2

**PRP:** platelet-rich plasma

**PS:** phosphatidylserine

**RBC Tr:** red blood cell transfusion in the last 3 months

**RBC:** red blood cell

**RDW:** red blood cell distribution width

**Retic:** reticulocytes

**RhD:** rhesus (Rh) system

**RNA:** ribonucleic acid

**ROS:** reactive oxygen species

**SD:** standard deviation

**SIRPα:** signal regulatory protein alpha

**SM:** sphingomyelin

**T:** patients being treated
Appendices – Research data management

RESEARCH DATA MANAGEMENT

The data obtained during my Ph.D. at the Radboud University Medical Center (Radboudumc) have been captured and stored on Labguru, a digital lab book client which is centrally stored and daily backed-up on the local Radboudumc server. Data was additionally backed-up on University servers belonging to the department of Biochemistry. All data archives (view only) are stored on Labguru and accessible by the associated senior staff members. The studies described in Chapter 3, 4, 5 and 6 were approved by the local Medical Ethical Committee (CMO Arnhem-Nijmegen) and was in accordance with the Declaration of Helsinki. The informed consent was obtained from patients and donors involved on the studies. Published data generated or analysed in this thesis are part of published articles and its additional files are available from the associated corresponding authors on request. To ensure interpretability of the data, all filenames, primary and secondary data, descriptive files and software used to provide the final results are documented along with the data.
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<td>2015</td>
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<td>The digital library for beginners.</td>
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<td>Endnote workshop.</td>
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<td>Poster presentation course.</td>
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<tr>
<td>Advanced conversation.</td>
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<td>Academic writing.</td>
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<td>0.3</td>
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<tr>
<td>Scientific integrity.</td>
<td>2016</td>
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<td>Presentation skills.</td>
<td>2016</td>
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<tr>
<td>Radboud Summer School – How to become an excellent lecturer.</td>
<td>2016</td>
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<tr>
<td>The art of Presenting Science.</td>
<td>2016</td>
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<tr>
<td>Within sight of my Ph.D.</td>
<td>2017</td>
<td>1.5</td>
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<tr>
<td>Achieving your goals and performing more successfully in your Ph.D.</td>
<td>2017</td>
<td>0.8</td>
</tr>
<tr>
<td>Career guidance for international Ph.D.’s.</td>
<td>2017</td>
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</tr>
<tr>
<td><strong>b) Seminars &amp; lectures</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Radboud Research Rounds</td>
<td>2015</td>
<td>3.3</td>
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<tr>
<td>Happier animal cage: happier scientist?</td>
<td>2015</td>
<td>3.3</td>
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<tr>
<td>More than add on Chemo selective Functionalization of Peptides and Proteins.</td>
<td>2015</td>
<td></td>
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<tr>
<td>Primary murine myoblasts of NDUFS4/ mice show normal differentiation and fusion.</td>
<td>2015</td>
<td></td>
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<tr>
<td>Construction of collagen scaffolds for cartilage regeneration.</td>
<td>2015</td>
<td></td>
</tr>
<tr>
<td>Targeting nanomedicines to epithelial barriers: Intestines, skin and lung.</td>
<td>2015</td>
<td></td>
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<tr>
<td>CPP nanobody conjugates as dual mode targeting legends for cancer cells.</td>
<td>2015</td>
<td></td>
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<tr>
<td>Calcium ORAI and killing: cytotoxic T lymphocyte effector function in a challenging tumor microenvironment.</td>
<td>2016</td>
<td></td>
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<tr>
<td>GFP complementation as functional assay for cytosolic peptide import.</td>
<td>2016</td>
<td></td>
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<tr>
<td>Polyplex formation the science of mixing Small molecules and skin regeneration.</td>
<td>2016</td>
<td></td>
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<tr>
<td>Optimize your flow cytometry – online event of BioRad company.</td>
<td>2016</td>
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<tr>
<td>Immune modulation through glycan coding of IgG.</td>
<td>2016</td>
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<tr>
<td>Live cell multispectral high content analysis of mitochondrial morpho function.</td>
<td>2016</td>
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<tr>
<td>Investigating the requirement of ankyrin of membrane.</td>
<td>2016</td>
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<tr>
<td>Glycosaminoglycans and lymphocytes, significant players in the ovarian cancer microenvironment.</td>
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<tr>
<td>Peptide uptake in 3D ovarian cancer models.</td>
<td>2016</td>
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<tr>
<td>Reactive oxygen species and Ion channel physiology.</td>
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<tr>
<td>Novel dynamic collagen scaffolds for urethral tissue engineering.</td>
<td>2016</td>
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<tr>
<td>Mitochondrial complex I inhibition triggers a mitophagy dependent</td>
<td>2017</td>
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</tr>
<tr>
<td>ROS increase leading to necroptosis and ferroptosis in melanoma cells.</td>
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</tr>
<tr>
<td>Event</td>
<td>Year</td>
<td>Hours</td>
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<tr>
<td>----------------------------------------------------------------------</td>
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<tr>
<td>Radboud Research Rounds: The Complement system meets the KIDNEY and the PATIENT</td>
<td>2017</td>
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<tr>
<td>Investigating engineered proteins: from cytosolic delivery in 2D to tumor penetration in 3D.</td>
<td>2017</td>
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<tr>
<td>Mesenchymal stem cells in emphysema: finding the right niche for alveolar repair.</td>
<td>2017</td>
<td></td>
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<tr>
<td>Getting a glimpse in tumor biology via RNA analysis.</td>
<td>2017</td>
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<tr>
<td>Sequencing of glycosaminoglycans with potential to interrogate sequence specific interactions.</td>
<td>2017</td>
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<tr>
<td>How mitochondrial (ultra)structure affects mitochondrial function.</td>
<td>2018</td>
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<tr>
<td>Delivery of oligonucleotides in ovarian cancer.</td>
<td>2018</td>
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<tr>
<td>Radboud Research Rounds: Peptide and protein conjugates for diagnostics and therapy. (<a href="https://www.ru.nl/imm/newsevents/immevents/generalevents0/radboudresearchroundsthemenanomedicine/">https://www.ru.nl/imm/newsevents/immevents/generalevents0/radboudresearchroundsthemenanomedicine/</a>)</td>
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<tr>
<td>Detection of 3O sulfated heparan sulfate.</td>
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<tr>
<td>Mitochondrial cell omics of Leigh Syndrome fibroblasts.</td>
<td>2018</td>
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<tr>
<td>Quantification of extracellular matrix proteins in COPD vs. control lung tissue by mass spectrometry.</td>
<td>2018</td>
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</tr>
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</table>

**c) Symposia & congresses**

<table>
<thead>
<tr>
<th>Event</th>
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<th>Hours</th>
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<tbody>
<tr>
<td>Radboud Frontiers: Radboud Frontiers in Cilia Medicine</td>
<td>2015</td>
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<tr>
<td>Ph.D. retreat (poster presentation).</td>
<td>2015</td>
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<tr>
<td>Radboud Science Day.</td>
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<tr>
<td>Ph.D. retreat (poster presentation).</td>
<td>2016</td>
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</tr>
<tr>
<td>Spetses Summer School (Spetses, Greece) – Molecular mechanisms of aging and regeneration (poster presentation).</td>
<td>2016</td>
<td>2.75</td>
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<tr>
<td>Ph.D. retreat (poster presentation).</td>
<td>2017</td>
<td>0.75</td>
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<tr>
<td>Radboud New Frontiers Symposium on the Microbiome</td>
<td>2017</td>
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<tr>
<td>21st European Red Cell Society – Erythrocyte Life Cycle: from stem cell to senescence (Heidelberg, Germany) (poster presentation).</td>
<td>2017</td>
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<tr>
<td>Ph.D. retreat (oral presentation).</td>
<td>2018</td>
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</table>

**d) Other**

<table>
<thead>
<tr>
<th>Event</th>
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<th>Hours</th>
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</thead>
<tbody>
<tr>
<td>Radboud Invites – RIMLS open day.</td>
<td>2018</td>
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</table>
TEACHING ACTIVITIES

e) Lecturing

<table>
<thead>
<tr>
<th>Activity</th>
<th>Year</th>
<th>Hours</th>
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<tbody>
<tr>
<td>Practical: skills and theoretical background in the 1RWL (Wet Lab Research) module for first year biomedical science students.</td>
<td>2015</td>
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<tr>
<td>Lecture: Introduction to Paroxysmal Nocturnal Hemoglobinuria</td>
<td>2016</td>
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<tr>
<td>Lecture: Erythrocyte Homeostasis in Patients with Paroxysmal Nocturnal Hemoglobinuria.</td>
<td>2016</td>
<td>0.1</td>
</tr>
<tr>
<td>Practical: Red blood cell Aging – flow cytometry.</td>
<td>2016</td>
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<tr>
<td>Practical: Anemia – flow cytometry.</td>
<td>2016</td>
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</tr>
<tr>
<td>Practical: skills and theoretical background in the 1RWL (Wet Lab Research) module for first year biomedical science students.</td>
<td>2016</td>
<td>1.3</td>
</tr>
<tr>
<td>Practical: Red blood cell Aging – flow cytometry.</td>
<td>2017</td>
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</tr>
<tr>
<td>Practical: skills and theoretical background in the 1RWL (Wet Lab Research) module for first year biomedical science students.</td>
<td>2017</td>
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</table>

f) Supervision of internships / other

<table>
<thead>
<tr>
<th>Supervision</th>
<th>Year</th>
<th>Hours</th>
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<tbody>
<tr>
<td>Supervision of a Master student on the project: Red Blood Cell Acetylcholinesterase: The Influence of the Membrane and Calcium on its Activity.</td>
<td>2016</td>
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<tr>
<td>Supervision master student on the project: Erythropoiesis in vitro.</td>
<td>2017</td>
<td>2</td>
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<tr>
<td>Supervision bachelor student on the project: Influence of mechanical stress on RBCs.</td>
<td>2018</td>
<td>4</td>
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</tbody>
</table>

**TOTAL** 42.5
ACKNOWLEDGEMENTS

In the past years, I have worked with pleasure on the research presented in this book. During this journey, the collaborative work of many people contributed to the ultimate success of this Ph.D. thesis. I would like to take this opportunity to thank all for having walked along by my side in this track.

The financial support given to me by the Brazilian government by the program Sciences without borders and the National Council for Scientific and Technological Development of Brazil (CNPq) allowed me to come to the Netherlands and perform high-quality research with excellent technical support together with top researchers. I am extremally grateful for the opportunity to enhance my scientific and personal skills, and I wish that such opportunities multiply and more Brazilians have the chance to empower theirs and others lives through education.

Dear Giel, once more, thank you for your guidance, help, and empathy. I thank you for being there back in January 2015, in that cold day with a warm welcoming talk on the train from Schiphol to Nijmegen, that was the beginning of our rewarding cooperation, it has been a pleasure working with you. Your care and understanding were essential in my adaption. I appreciate all the fruitful meetings, your insights, the beautiful stories, the draws, the schemes, the discussions, and your eagerness in sharing knowledge. I have always valued your contagious enthusiasm for sciences and the space you gave me to shape myself my way of thinking. I am extremally thankful for your availability and supervision in these countless hours you have spent in my scientific development.

Dear Merel, your advice was fundamental in this path. Thank you for applying the detailed and practical mindset to our weekly meetings and discussions, they surely enriched my scientific skills.

Dear Roland, thank you for the opportunity to be part of your lab and for your precious insights during presentations and manuscripts writing.

I would like to thank my Biochemistry Department (former) colleagues and friends Corina, Dan, Dennis, Dirk, Elianne, Estel, Judith, Jürgen, Krissie, Lisette, Marco, Maryia, Mehdjabin, Petra, Philip, Rike, Samuel, Sander, Sjors, Valentina, William and Wouter for creating such an amazing atmosphere in the lab, pleasant talks during lunch, and all the fun. Judith, thank you for mentoring me in the first months of my
experience in the lab. Jenny, thank you for your help with experiments, for maintaining the lab organization and for so many talks. Dear Lisanne, thank you for sharing the happiness and disappointments of a Ph.D. track, you’re the best neighbor ever. Amigos, Omar e Cris, obrigado por compartilhar essa jornada e por serem suporte dentro e fora do lab.

I want to thank all the project collaborators in our red blood cell studies.

Science aims for improvements for the next generations, I thank Luna, Maria Júlia, Moos, Nala, Sam, and Vitória for motivating the scientific work of mine and many more.

Away from my country, I created new and strong bonds. I appreciate the friendship of all my Dutch friends. Bram, Igor, Iris, Lisanne, Loek, Marije, Petra, thank you for welcoming me to your lives, for the talks, dinners, game-nights, gezelligheid and for have been encouraging me to “Nederlands leren” (please do not give up).

Querida marida Ana, você foi primeiro porto seguro que encontrei num ambiente novo e ver no outro a semelhança de vida, trocar confidencias, rir, aprender, viajar e morar junto contribuíram pra fortalecer essa amizade que prezo tanto. Obrigado por ser tão carinhosa e amável, quero você sempre por perto.

Queridinhos, vocês trazem o Brasil pra essa aventura. Bia, espero que você responda a mensagem de 2016 no grupo. Obrigado por ser tão leve e empática, por ensinar como entender o próximo. Você traz o balanço do grupo mesmo sem saber de nada. E Edu, a gente q tem prótese a gente sente, né? Obrigado por dividir a casa e a experiência do doutorado, por ensinar como aproveitar os momentos, por compartilhar frustações e experiências de vida.

Queridinha Angélica, você exala alegria, goodvibes e desorientação geográfica. Obrigado por ser suporte, amiga, housemate, hoster, colega, confidente, militante, minha paraninfa e me ensinar que a vida é pra ser vivida, sem arrependimentos, sem preconceitos, livre e confiante.

A aventura começou virtualmente, e até hoje estamos compartilhando experiências em diferentes partes do mundo. Isa, a gente se deu tão bem que saímos do virtual e passamos pro real. Obrigado por ser amiga e por estar sempre disponível.
Minha paraninha **Thinitis**, você é força e superação. Obrigado pela coragem, por mostrar o quão possível é pra quem acredita e corre atrás do sonho. Agradeço pela amizade, pelas noites de filme, pelas aventuras na Alemanha, pelas comidas deliciosas, pelas comprinhas baratinhas e descontos. Só love aqui.

Meus amigos do Brasil: **André, Anielle, Bruno, Henrique, Joás, Manu e Rafena** obrigado por torcerem por mim, saibam que é reciproco. **Gleice (in memoriam)**, nea, obrigado por tudo, por me mostrar que o importante é simplesmente ser do jeito que você é e se orgulhar disso. Eu os amo e sei que você estão aqui comigo em cada conquista.

**Vó, Vô, tios, tias e primos**, obrigado por todo o amor. **Tia Fatinha**, obrigado por acreditar e encorajar. **Tia Pretinha**, obrigado pelo cuidado e entendimento. Primos, as oportunidades aparecem para serem usufruídas, acreditem em vocês e façam seu melhor, obrigado pela torcida.

Agradeço minha família por moldar meu caráter. **Pai e mãe**, obrigado pelo apoio, por estar sempre presentes, por serem amigos, por serem motivadores, por serem professores. A vida é um aprendizado constante, a jornada das relações pode ser dolorosa, porém compartilhar amor tanto pra si quanto pro próximo é a melhor lição que podemos aprender. Meus irmãos, **Hanna, Jovane e Liliane** obrigado por dividir momentos e por serem quem são, amo vocês.

Lovely **Family Benthem**, thank you for embrace me. It is such a blessing being surrounded by so much affection and respect. Thank you **Jochem, Maarten, Sandra and Sam** for all the pleasant moments, gourmetten, Chinese food, Sinterklaas and Christmas fun nights. **Albert and Ria**, thank you especially for your kindness and caring.

**Tijmen**, I have extreme admiration for the person you are therefore I have extreme gratitude for having you in my life. Thank you for your support, for your understanding, to be open to hear and to comfort. You make me better, fearless, prouder and humbler person. I wish that our collaboration in life persists successfully as always has been. I love you… more.

Bedankt allemaal!
Curriculum Vitae

Joames Kauffmann Freitas Leal is born on January 15th, 1987 in Baturité, Ceará, Brazil. He moved to Fortaleza to attend high school, and afterward joined the Computer Sciences bachelor program at the Universidade Estadual do Ceará. Finding out his Life sciences calling, in 2007, he began the Pharmaceutical sciences studies at the Universidade Federal do Ceará, where he performed significant research during his tutor and intern activities while becoming a pharmacist throughout the 5 years bachelor program. Within the Laboratory of Parasitology, he implemented educational actions in communities under schistosomiasis infection hazard and executed his master project aiming to evaluate a new diagnostic tool for schistosomiasis in low-endemic areas. After obtaining his M.Sc. degree in Medical Pathology, he lectured Clinical Hematology and employed his pharmacist skills in developing pharmaceutical products and in applying pharmaceutical care until late 2014. In January of 2015, he crossed the ocean to perform his Ph.D. in Nijmegen, the Netherlands, supported by the Sciences without Borders fellowship program – CNPq awarded by the Brazilian Ministry of Education. In the Radboud Institute for Molecular Life Sciences, Department of Biochemistry, using molecular and cell biology tools to investigate red blood cells homeostasis under various physiological and non-physiological conditions, he successfully gathered the scientific data shown in this thesis.
IN V I T A T I O N
for attending the public
defense of the doctoral thesis:
RED BLOOD CELL STRESS: A CHALLENGE TO HOMEOSTASIS
JOAMES KAUFFIMANN FREITAS LEAL
joames.leal@gmail.com
Monday, November 18, 2019,
10:30, in the Aula
Paranymphs
Reception on-site afterward
Your presence would be much appreciated