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Evaluation of biochemical effects of oxidative stress in blood bank-stored red blood cells

PhD student

Cristiana Mirasole

Coordinator

Prof. Giorgio Prantera

Tutor

Prof.ssa AnnaMaria Timperio

A te, Nonno Renzo!

AIM OF THE STUDY

Blood banking underpins modern medical care, but blood storage, necessary for testing and inventory management, reduces the safety and efficacy of individual units of red blood cells (RBCs). Stored RBCs are damaged by the accumulation of their own waste products, by enzymatic and oxidative injury. These chemical activities lead to a complex RBC storage lesion that includes haemolysis, reduced *in vivo* recovery, energy and membrane loss, altered oxygen release, reduced adenosine triphosphate and nitric oxide secretion, and shedding of toxic products. The latter includes also free iron that can potentiate susceptibility to oxidative stress, which in turn promotes oxidative-lesions to proteins, lipids (peroxidation) and morphological changes (membrane blebbing, vesiculation). In particular, reactive oxygen species seem to be the eligible trigger for these lesions and the main contributor to the final quality loss (both at the macroscopic and microscopic levels) of RBCs.

In order to ensure safety and quality of blood products, the study of *in vitro* (storage conditions) ageing of red blood cells has recently taken advantage of the introduction of mass spectrometry-based “omics” disciplines, such as proteomics, metabolomics and lipidomics, which are characterized by the systematic determination and quantification of broad classes of molecules, such as proteins, metabolites and lipids. Data from multiple “omics” platforms can be then integrated through bioinformatic approaches and mathematical modeling to obtain a system biology level of understanding, which has been performed during our research.

During this work of thesis, we focused on the evaluation of irreparably protein oxidative modifications, in particular those related to non-enzymatic glycosylation of hemoglobin α and β -chains (HbA1c). The observation of apparently increased rates of HbA1c levels over storage progression might be also exacerbated by the excessive glucose found in anticoagulant and additive solutions. On the contrary, we followed how some of these oxidation products can be repaired (e.g. oxidations of sulfur-containing amino acid residues), representing an exceptional way to sense the redox state in cells. The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been found to regulate its activity in response to storage-induced oxidative stress through the formation of an intramolecular disulfide bond involving catalytic cysteines. This mechanism has been postulated to function as a metabolic switch to redirect

carbohydrate flux from glycolysis to the pentose phosphate shunt, allowing the red cell to maintain oxidation-reduction balance and protect itself against oxidative insults. Hereby, we describe alternative storage protocols which have been proposed in order to overpass these hurdles, such as RBC anaerobic storage. We could confirm previous evidences about long term anaerobiosis promoting glycolytic metabolism in RBCs and prolonging the conservation of high energy phosphate reservoirs and purine homeostasis. In parallel, we evidenced that, contrarily to aerobic storage, anaerobiosis impairs erythrocyte capacity to cope with oxidative stress by blocking metabolic diversion towards the pentose phosphate pathway, which negatively affects glutathione homeostasis. Therefore, although oxidative stress was less sustained than in aerobically stored counterparts, its markers still accumulate over anaerobic storage progression.

Finally, we presented an HPLC-microTOF-Q approach to investigate the RBC lipidome. We could exploit this analytical workflow to consolidate existing knowledge on the RBC lipid composition and individuate statistically significant fluctuations of lipids throughout storage duration of RBC concentrates under blood bank conditions. While this field of research still warrants future investigations, our analysis indicated ceramides, glycerophospholipids and sterols as key targets of RBC storage lesions to the lipidome, which will deserve further targeted investigations in the future.

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CHAPTER 1: INTRODUCTION

1.1 ALTERATIONS TO RBCs DURING STORAGE (“THE STORAGE LESIONS”)

1.1.1 Why Red Blood Cells?

Derived from pluripotent stem cells in bone marrow through a maturation process called erythropoiesis, mature Red Blood Cells (RBCs) are biconcave disks of approximately 7.2 μm in diameter, 1.5 to 2.5 μm thick, with a mean volume of 90 fL. Along the developmental process, there is a reduction in cell volume, condensation of chromatin, loss of nucleoli, decrease in the nucleus, RNA, mitochondria, and an increase in hemoglobin synthesis, resulting in a mature RBC, which lacks a nucleus and organelles. The primary function of RBCs is to transport oxygen from the lungs to the body tissues, where the exchange for carbon dioxide is facilitated through synergistic effects of hemoglobin, carbonic anhydrase, and band 3 protein, followed by carbon dioxide delivery to the lungs for release. Successful oxygen transport is dependent on efficacy of the three elements of RBC metabolism: the RBC membrane, hemoglobin, and cellular energy. Continuous research of oxygen transport is crucial for development of improved RBC storage and biopreservation technologies.

1.1.2 History of Blood storage: its meaning for Transfusion

Whole blood storage was first demonstrated by Robertson in 1917. Acid-citrate-dextrose (ACD) and Citrate-phosphate-dextrose solution (CPD) were subsequently approved for 21-day storage of blood. CPD with adenine (CPDA-1) was later introduced and used for extending the shelf-life of stored blood for up to 5 weeks. Red blood cells stored in these solutions have shown steady deterioration after about 5 to 6 weeks as determined by the inability of such cells to survive in the circulation for 24 hours after reinfusion back into the human donor. It has been observed that during continued refrigerated storage, glucose is consumed at a decreasing rate, as the concentration of metabolic waste, i.e. lactic acid and hydrogen ions, increases. Such a decrease in the rate of glucose metabolism leads to depletion of adenosine triphosphate (ATP) which directly correlates to the recovery of RBCs when the cells are returned to the circulation. The development of additive solutions for the preservation of red blood cells after their separation from whole blood has allowed the design of formulations which are specifically tailored to the needs of RBCs. Additive solutions such as Adsol®

(AS-1), Nutricel® (AS-3), Optisol® (AS-5), and Erythro-Sol® were designed to extend the storage of RBCs at 4°C.

In Italy, the “design” of the national blood system was changed through the "New discipline for blood transfusion activities and national production of blood derivatives" [Italian Parliament, October 21st, 2005]. In parallel, the 2002/98/EC directive of the Council of Europe and the 2004/33/EC, 2005/61/EC and 2005/62/EC directives of the European Commission have been transposed into national laws. Actually, new regulatory standards have been introduced, contributing to improve the ability to store RBCs and to rich the strategic goals of the Italian blood system.

At present, transfusion of RBCs is a common therapeutic intervention for which recent developments suggest steps that might be taken to improve their quality, safety and their benefits for the transfused recipient. Most RBC products are derived by collection of 450-500 ($\pm 10\%$) mL of whole blood into anticoagulant solutions (typically citrate-dextrose-phosphate) from volunteer donors and removal of the plasma by centrifugation (see Table 1) and, in some cases, also leukocytes. Although in healthy subjects, most RBCs live for 110 to 120 days (with the standard deviation of their life span of about 6 days in normal subjects [Högman et al, 1999], after removal of the plasma, the resulting product is red blood cells (referred to informally as “packed red blood cells”) with 42-day blood bank shelf life, which are stored at $4 \pm 2^\circ \text{C}$ in a slightly hypertonic additive sterilely solution, generally SAGM (sodium, adenine, glucose, mannitol, 376 mOsm/L).

Table 1- Special processing of RBC for transfusion

Process	Indications	Technical Considerations
Leucocytes reduction	Decreased risk of recurrent febrile, nonhemolytic transfusion reactions Decreased risk of cytomegalovirus transmission Decreased risk of HLA-alloimmunization	Most commonly achieved by filtration, usually soon after collection (pre-storage)
Washing (removes residual plasma)	Decreased risk of anaphylaxis in IgA-deficient patients with anti-IgA antibodies Decreased reactions in patients with history of recurrent, severe allergic or anaphylactoid reactions to blood product transfusion	Wash fluid is 0.9% NaCl±dextrose May lose 20% of red blood cells in washing process

The RBC is a highly specialized cell; because it lacks a nucleus and other cellular machinery, it has to rely on its original contents of enzymes and other proteins for the whole of its limited lifetime. In addition, because it lacks mitochondria, it cannot use oxidative phosphorylation for adenosine triphosphate regeneration.

In term of prolonged storage, this means that RBCs undergo a dramatic molecular changes, the so-called "red blood cell storage lesion"[Hess 2006, Hess 2007,Hess 2010]. The RBC storage lesions include:

i. *Metabolic changes:*

1. RBCs reduce concentrations of adenosine 5'-triphosphate (ATP) and 2,3-diphosphoglycerate (2,3-DPG). Glycolysis is the only source of energy for RBCs, through which they break down glucose to make ATP and produce lactic acid and protons as a result. The protons produced in glycolysis make the storage solution and the red cells increasingly acidotic. This acidosis in turn slows further glycolysis because the protons inhibit hexose kinase and phosphofructokinase, the first two activating enzymes in the main glycolytic pathway, so that less ATP is made as storage progresses [Hess 2006, Hess 2007, Hess 2010, Ian Chin-Yee et al., 1997].
2. The pH typically decreases from 7.0 to 6.5, while the rate of glucose consumption fall down 50%, starting from RBCs collection from venous blood at a pH of about 7.35, put into CPD at a pH of 5.5 to 5.8 [Hess 2006].
3. The fluxes of sodium ions (massive entry into the cell) and potassium ions (exit from the cell) are involved in the storage lesion, because of loss of function (usually transient) of cation pumps , since the Na^+/K^+ pump is inactive at 4°C [Bennett-Guerrero et al., 2007] and ATP-dependent. This leads to loss of intracellular potassium and accumulation of sodium within the cytoplasm. The concentration of extracellular potassium in the blood bag typically accumulates at a rate of about 1 mEq/day, causing damages when the blood is used in cardiac bypass machines [Hall et al., 1993].

ii. *Enzymatic changes:*

Enzymatic damages are related to the presence of white blood cells (WBCs) during storage period. Indeed, it has been demonstrated that WBCs break down and release enzymes such as proteases, lipases and glycosidases in the storage solution. These enzymes attach their target on the outer surface of erythrocytes [Riedner et al., 1990]. Proteases can contribute to the loss of protein antigen strength with storage and generally they are blocked by plasma antiproteases. Lipases such as phospholipase can dealkylate trialkyl-glycerols to produce lysophospholipids. An important example of this process is the production of platelet activating factor (PAF), thought to be responsible for some cases of transfusion-related acute lung injury (TRALI) [Gajic et al., 2007]. Glycosidases remove sugars from glycolipids and glycoproteins, and the loss of these sugars can expose underlying structures that increase the binding of stored red cells to endothelial cells [Sparrow et al., 2007]. The removal of the WBCs by filtration, the so called leukoreduction, can reduce enzymatic damage, thereby improving recovery and decreasing the hemolysis of stored red cells [Heaton et al., 1994]. The discovery of TRALI was the first and still the most important example of possible damages associated with stored blood. About glycosidases, Sparrow's group [Sparrow et al., 2007] has shown that stored red cells adhere to endothelial cell monolayer proportionally with the loss of membrane sugars. Such adherence is assumed to be responsible of endothelial inflammation if the red cells subsequently break down and release iron and heme. For all these reasons, leukoreduction increases red cell recovery by about 2% and reduces the hemolysis by half at the end of the storage.

iii. *Shape change and membrane vesiculation:*

RBC storage, even for relatively short periods of time (between the 7th and the 14th day of storage [Tamara, Berezina et al, 2002]), affects RBC morphology and function (Table 2). The echinocyte-spherocytocyte red blood cell shape transformation [Lockwood et al., 2003] is induced by loss of membrane through the blebbing of vesicles from the spicules of cells that have undergone echinocytic (in the early stage). After prolonged storage (beyond 21 days), when

the ATP-content of stored RBC decreases rapidly, the total amount of released vesicular proteins increases exponentially, reaching 10 times the amount by 50 days as compared to 14 days of storage. The vesiculation contributes to irreversible cell shape and membrane changes, in order to eliminate proteins and lipids that have been altered by oxidative stress, as to protect the cell from a further chain reaction of stress and consequent removal from the circulation [Willekens et al., 2008]. It can be considered not only part of the physiological process of maturation and programmed cell-death mechanism unmasked by low concentrations of ATP, but also the result of a cell-death-independent step making vesicles that carry away oxidized lipids and damaged proteins [Hess 2010, Salzer et al., 2008]. Moreover, the shape change seen during RBC storage is associated with rheologic changes, increased viscosity and reduced flow in capillary systems [Relevy et al., 2008].

Table 2- Changes in RBC shape during storage [Berezina et al., 2002]

Day of storage	Shape RBC		
	Discocyte (%)	Reversibly changed RBC (%)	Irreversibly changed RBC (%)
5 th	79.0±2.7	14.0±1.7	7.0±1.6
7 th	78.0±3.0	13.6±1.7	8.4±1.6
14 th	57.4±3.8*^	27.9±1.9*^	14.7±2.6*^
21 th	53.7±3.5*	30.6±3.0*	15.7±3.3*
28 th	47.6±3.6*	35.2±1.6*^	17.2±4.1*^
35 th	37.5±5.0*^	40.6±3.4*^	21.9±5.0*
42 th	23.3±4.3*^	46.8±6.7*	29.9±4.0*^

* P< 0.01 vs 5th day

^ P< 0.01 vs previous point

iv. *Oxidative damage:*

- RBC oxidant susceptibility during storage results in Reactive Oxygen Species (ROS) targeting protein and lipid fractions [Dumaswala et. al., 1999]. Lipid peroxidation and phospholipid reorganisation of the membrane occurred in the first 7-14 days of cold storage [Brad et al., 2012]. Banked RBCs show a time-dependent increase in (i) protein clustering [D'Amici et al., 2007, Walpurgis et

al., 2012], especially on band 3 (early, in the first 7 days of storage); (ii) carbonyl modification of band 4.1 [D'Alessandro et al., 2012, Kriebardis et al., 2007] and changes in spectrin-protein 4.1-actin interactions [Card 1988, Wolfe et al., 1986]; (iii) malondialdehyde increase and (iv) a decline of erythrocyte glutathione (GSH) levels and glutathione-peroxidase (GSH-PX) activity [Umakant et al., 1999]. In contrast loss of acetylcholinesterase activity and haemolysis of RBC occurred relatively late (14-21 days) during the storage period [Brad et al., 2012]. Microvesicle generation from ATP-depleted RBC also correlates with the breakdown of polyphosphoinositides to diacylglycerol on the inner monolayer [Müller et al., 1981]. Membrane budding is dependent on both the diffusible diacylglycerol partitions into the outer monolayer and exoplasmic exposure of phosphatidylserine (PS), a negatively charged phospholipid. Microvesicles (160 nm diameter) from RBC stored for more than 20 days [Kriebardis et al., 2008] contain band 3, are enriched in acetylcholinesterase and in stomatin, but threefold depleted of flotillin 2 that remains in the residual RBC membrane [Salzer et al., 2008]. Microvesicles from long stored RBC contain heavily aggregated hemoglobin, band 3 aggregates and increasing amounts of autologous IgG [Kriebardis et al., 2008], while the residual RBCs reveal a decrease in band 3 content, but yet a substantial increase in bound IgG [Kriebardis et al., 2007].

- The band 3, in addition of being an anion exchanger protein, is the site of macromolecular assembly for cytoskeleton anchorage, glycolytic enzymes (the N-terminal cytosolic domain of band 3 is an inhibitory docking site for glycolytic enzymes), and regulatory proteins. Indeed, band 3 is involved in the modulation of oxygen transport by influencing the intracellular pH (and thus oxygen off-loading) through its regulation of the chloride shift (exchange of Cl^- / HCO_3^- anions).
- Under oxidative stress, haemoglobin (Hb) could be oxidized to met-Hb (a protein unable to carry oxygen) which can be converted into low-spin ferric Hb compounds named hemichromes, a variant of Hb in which cystein thiol groups have been dangerously oxidized to form denatured Hb-aggregates precipitating in inclusion bodies within RBCs, also known as “Heinz bodies”. Normally,

reversible hemichromes can be reduced back to deoxyHb [Telen et al., 2004]. Significant distortions in the tertiary structure of the Hb can lead to the so-called ‘irreversible hemichromes’. The latter cannot be converted back to normal Hb [Rachmilewitz et al., 1971] and are susceptible to further oxidative injury, during prolonged storage, which may result in their fragmentation and formation of aggregates.

- Oxidation to proteins also results in the progressive accumulation of glycated forms of haemoglobin (Hb1Ac, non-enzymatic addition of sugar moieties to the proteins) during storage duration in the membrane and cytosol [Sparrow et al., 2007]. Notably, altered membrane protein glycosylation patterns might influence the rheological properties of RBCs [Berezina et al., 2002].

1.2 PARAMETERS OF THE OXIDATION/REDUCTION BALANCE IN *in vivo* RED BLOOD CELLS

Red blood cells are responsible *in vivo*, for oxygen transport from lung to tissues. Their function depends on reduced state of iron (Fe^{2+}) in hemoglobin (Hb). Both element oxygen and iron are able to quickly shift their oxidative state in response to different emerging stimuli. Literature shows that many approaches for evaluation of RBC redox status have been established, including indirect approaches such as measurement of protein oxidation, that reflects an accumulated damage [Lee, Britz-McKibbin, 2009]. The bigger the reactive species release is the higher normal deformability and flexibility of erythrocytes is disturbed.

1.2.1. Reactive species generation

In erythrocyte, oxygen (O_2), nitrogen oxide ($\text{NO}\bullet$) and iron (Fe^{2+}) are found together. All these elements act in normal conditions in established mechanisms but they may generate alone or together reactive species, named radicals, that damage red blood cells as well as vascular endothelium (Figure 1).

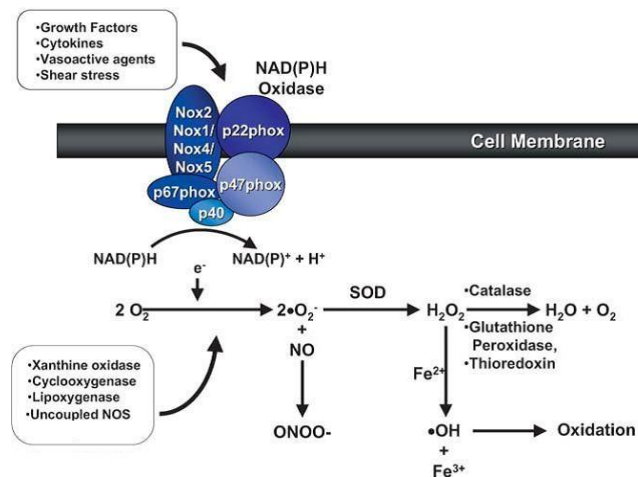


Figure 1-Generation of superoxide ($\bullet\text{O}_2^-$) and H_2O_2 from O_2 in vascular cells. Many enzyme systems, including NAD(P)H oxidase, xanthine oxidase, and uncoupled nitric oxide synthase (NOS) among others, have the potential to generate reactive oxygen species (ROS). Superoxide acts either as an oxidizing agent, where it is reduced to H_2O_2 by superoxide dismutase (SOD), or as a reducing agent, where it donates its extra electron (e^-) to form ONOO^- with NO. Hydrogen peroxide is scavenged by catalase, glutathione and thioredoxin systems, and can also be reduced to generate $\bullet\text{OH}$ in the presence of Fe^{2+} [Touyz et al., 2004].

A radical is a chemical species that possesses a single unpaired electron in outer orbitals, is able to independently exist and is also known as free radical. Radicals are highly reactive in extracting an electron from any neighbor molecule in order to complete their own orbitals.

There are two main groups of free radicals: ROS or reactive species of oxygen and RNS or reactive nitrogen species. ROS and RNS can act together damaging cells and causing nitrosative stress. Therefore, these two species are often collectively referred to as ROS/RNS.

1.2.2. Reactive oxygen species

Reactive species of oxygen refers to a group of highly reactive O_2 metabolites, including superoxide anion ($\text{O}_2^{\bullet-}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1\text{O}_2$), and hydroxyl radical ($\text{OH}\bullet$), that can be formed within cells. Reactive oxygen species are constantly formed as byproducts in normal enzymatic reaction in all human cells through normal aerobic processes as mitochondrial oxidative phosphorylation or as necessary products in neutrophils, in order to kill invading pathogens. The above mentioned phenomena are consuming oxygen processes. Erythrocytes must save oxygen for delivering it to the cells; as a consequence red blood cells lack

mitochondrion the main oxygen consumer within cell. In this particular condition the source of ROS in erythrocyte may be the carried oxygen itself.

Within cells where transitional metals are bounded to proteins (in metal containing proteins or enzymes) oxidative attack of O_2 tend to be slow, meaning that a first single electron is relatively difficult to add. As a consequence superoxid radical ($O_2^{\bullet -}$) will form very slow. In the presence of a free electron, the univalent reduction of oxygen yields reactive species of oxygen. (Figure 2).

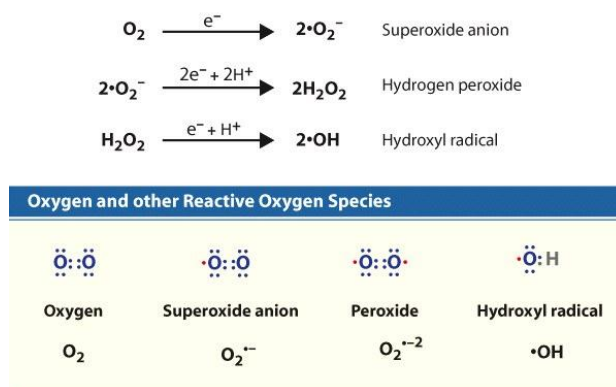
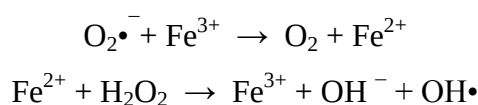


Figure 2-Several reactive oxygen species that are generated in cells are shown. The most damaging radical is the hydroxyl radical.

Superoxide has an unpaired electron, which imparts high reactivity and renders it unstable and short-lived. Superoxide is water soluble and acts either as a reducing agent, where it donates its extra electron to form $ONOO^-$ with NO [Darley-Usmar et al. 1995; Fridovich 1997], or as an oxidizing agent, where it is reduced to H_2O_2 . The latest, in the next one-electron reduction step, generates water and the hydroxyl radical ($OH\bullet$) which is probably the most reactive free radical. A final electron acceptance reduces hydroxyl radical to water.

Superoxide radical ($O_2^{\bullet -}$) is a reactive radical, however it cannot diffuse to far having limited lipid solubility. Instead it might react in the presence of ferric iron (Fe^{2+}) with dehydrogen peroxide generating the most potent hydroxyl radical through a non-enzymatic reaction known as Haber-Weiss reaction. The reaction takes place in two steps that involve ferric iron and superoxide as follows:

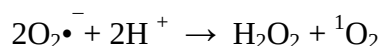


The general non-enzymatic reaction occurring in living cells is:



Hydrogen peroxide (H_2O_2) is not a free radical, but is produced mainly from dismutation of $\text{O}_2\bullet^-$. H_2O_2 is lipid soluble and as a consequence it can diffuse through lipid membranes.

Another reactive species (but not a radical) derived from molecular oxygen is singlet oxygen, designated as $^1\text{O}_2$. Singlet oxygen ($^1\text{O}_2$), a highly excited state created when molecular oxygen absorbs sufficient energy to shift an unpaired electron to a higher orbital, can be formed from superoxide radical:



Singlet oxygen is even more reactive than the hydroxyl radical, although it is not a radical. As a conclusion the most reactive radical is hydroxyl ($\text{OH}\bullet$) which indiscriminately extracts electrons from any other molecules around it, whereas superoxide ($\text{O}_2\bullet^-$) and hydrogen peroxide (H_2O_2) are more selective in their reactions with biological molecules.

All the above reactions and processes take place in all human cells including erythrocytes. These pro-oxidants are tightly regulated by anti-oxidants such as superoxide dismutase, catalase, thioredoxin, glutathione, anti-oxidant vitamins, and other small molecules [Stralin et al., 1995; Halliwell 1999; Channon and Guzik, 2002; Yamawaki et al., 2003].

Under normal conditions, the rate of ROS production is balanced by the rate of elimination. However, a mismatch between ROS formation and the ability to defend against them by antioxidants results in increased bioavailability of ROS leading to a state of oxidative stress [Griendling et al., 2000; Landmesser and Harrison, 2001; Zalba et al., 2001].

1.3 OXIDATIVE STRESS DURING THE FORMATION OF STORAGE LESIONS IN BLOOD BANKING RBCs: CHEMICAL MODIFICATION OF PROTEINS BY REACTIVE OXYGEN SPECIES

Protein oxidation is the covalent modification of a protein induced either directly by reactive oxygen species (ROS) or indirectly by reaction with secondary by-products of oxidative stress [Shacter, 2000, Dean et al., 1997]. Circulating RBCs are equipped, *in vivo*, with effective anti-oxidative systems that make them mobile free radical scavengers, providing antioxidant protection not only to themselves but also to other tissues and organs in the body [Siems, 2000, Arbos et al., 2008]. A condition of oxidative stress develops when the critical balance between oxidants and antioxidants is disrupted due to depletion of antioxidants and/or excess accumulation of ROS [Dalton et al., 1999]. Indeed, the irreversible events occurring during the storage process, exemplified by the haemolysis in the second half of the actual maximal blood bank storage period, are mostly activated by radical species generated by prolonged, continuous oxidative stress [Wolfe 1986, Racek et al., 2001].

Because there are so many mechanisms for induction of protein oxidation and because all of the amino-acyl side chains can become oxidatively modified, there are numerous different types of protein oxidative modifications (Table 3).

The demonstration that oxidatively modified forms of proteins accumulate during storage and oxidative stress, has focused attention on physiological and non-physiological mechanisms for the generation of ROS and on the modification of biological molecules by various kinds of ROS.

Table 3-Oxidative Modifications of Proteins [Shacter 2000]

Modification	Amino acids involved	Oxidizing source^a
Disulfides, glutathiolation	Cys	All ONOO ⁻
Methionine sulfoxide	Met	All, ONOO ⁻
Carbonyls (aldehydes, ketones)	All (Lys, Arg, Pro, Thr)	All
Oxo-histidine	His	γ -Ray, MCO, ¹ O ₂
Dityrosine	Tyr	γ -Ray, MCO, ¹ O ₂
Chlorotyrosine	Tyr	HOCl
Nitrotyrosine	Tyr	ONOO ⁻

[continued]

Tryptophanyl modifications, (N-formyl)kynurenine	Trp	γ -Ray
Hydro(pero)xy derivatives	Val, Leu, Tyr, Trp	γ -Ray
Chloramines, deamination	Lys	HOCl
Lipid peroxidation adducts (MDA, HNE, acrolein)	Lys, Cys, His	γ -Ray, MCO (not HOCl)
Amino acid oxidation adducts	Lys, Cys, His	HOCl
Glycoxidation adducts	Lys	Glucose
Cross-links, aggregates, fragments	Several	All

^a MCO-metal catalyzed oxidation; All = γ -ray, MCO, HOCl, ozone, $^1\text{O}_2$.

The pioneering studies of Swallow, Garrison, and Scheussler and Schilling demonstrated that reactions with $\text{OH}\cdot$ mainly initiated the modification of proteins; however, the course of the oxidation process is determined by the availability of O_2 and $\text{O}_2^{\cdot-}$ or its protonated form ($\text{HO}_2\cdot$). Collectively, the chemical effects of oxidative changes on membrane proteins can lead to diverse functional consequences, such as oxidation of amino acid residue side chains, formation of protein-protein cross-linkages, and oxidation of the protein backbone resulting in protein fragmentation (Figure 3).

Current concepts of ROS signaling can be divided into two general mechanisms of action: 1) alterations in the intracellular redox state, and 2) oxidative modifications of proteins. The first is due to the alteration of the “redox-buffering” capacity of intracellular thiols, primarily GSH and thioredoxin (TRX), maintained by the activity of GSH reductase and TRX reductase, respectively.

Both of these thiol redox systems counteract intracellular oxidative stress by reducing both H_2O_2 and lipid peroxides, reactions that are catalyzed by peroxidases (e.g., GSH peroxidase catalyzes the reaction $\text{H}_2\text{O}_2 + 2\text{GSH} \rightarrow 2\text{H}_2\text{O} + \text{GSSG}$). The second is the subject of discussion below.

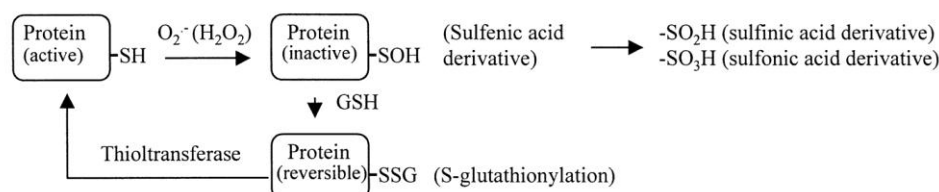
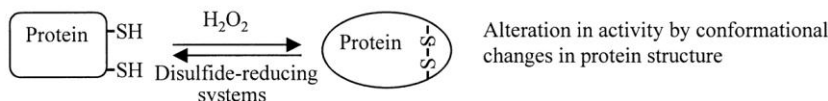
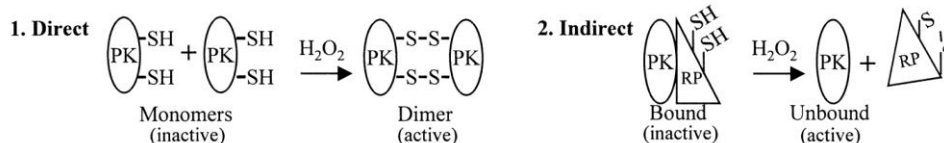
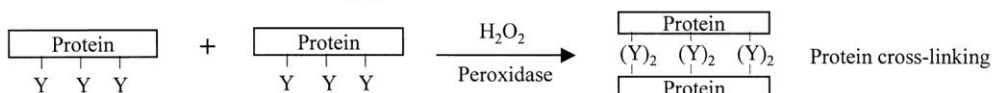
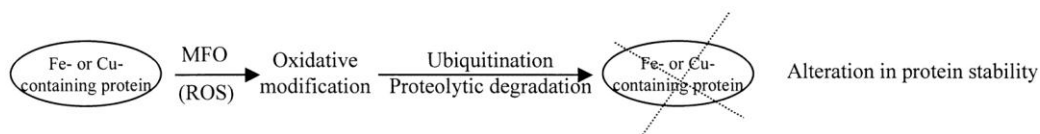
A MODIFICATION OF PROTEINS BY OXIDATION OF CYSTEINE RESIDUES**B FORMATION OF INTRA-MOLECULAR DISULFIDE LINKAGES****C PROTEIN DIMERIZATION BY INTER-MOLECULAR DISULFIDE LINKAGES****D DITYROSINE FORMATION BY H₂O₂/PEROXIDASE-DEPENDENT REACTIONS****E METAL-CATALYZED OXIDATION OF PROTEINS BY “FENTON-LIKE” CHEMISTRY**

Figure 3-Oxidative modification of proteins. A: the sulfhydryl (-SH) group of cysteine residues in proteins may be modified by $O_2^{\bullet-}$ (or H_2O_2) to form various oxidized derivatives. S-glutathionylation appears to confer reversibility to such modifications by the action of thioltransferases. B: formation of intramolecular disulfide bridges can alter protein activity by changes in conformation. C: intermolecular disulfide linkages can mediate protein dimerization. D: H_2O_2 - or peroxidase-catalyzed dityrosine formation can induce protein cross-linking. E: transitional metal-containing proteins may be targets of site-directed, metal-catalyzed oxidation by ROS produced by certain mixed-function oxidases (MFO), which “target” them for ubiquitination and degradation by proteases. This is a potential mechanism for ROS-mediated alterations in protein stability [Thannickal et al., 2000].

1.3.1 Oxidation of Sulfur-containing Amino Acid Residues

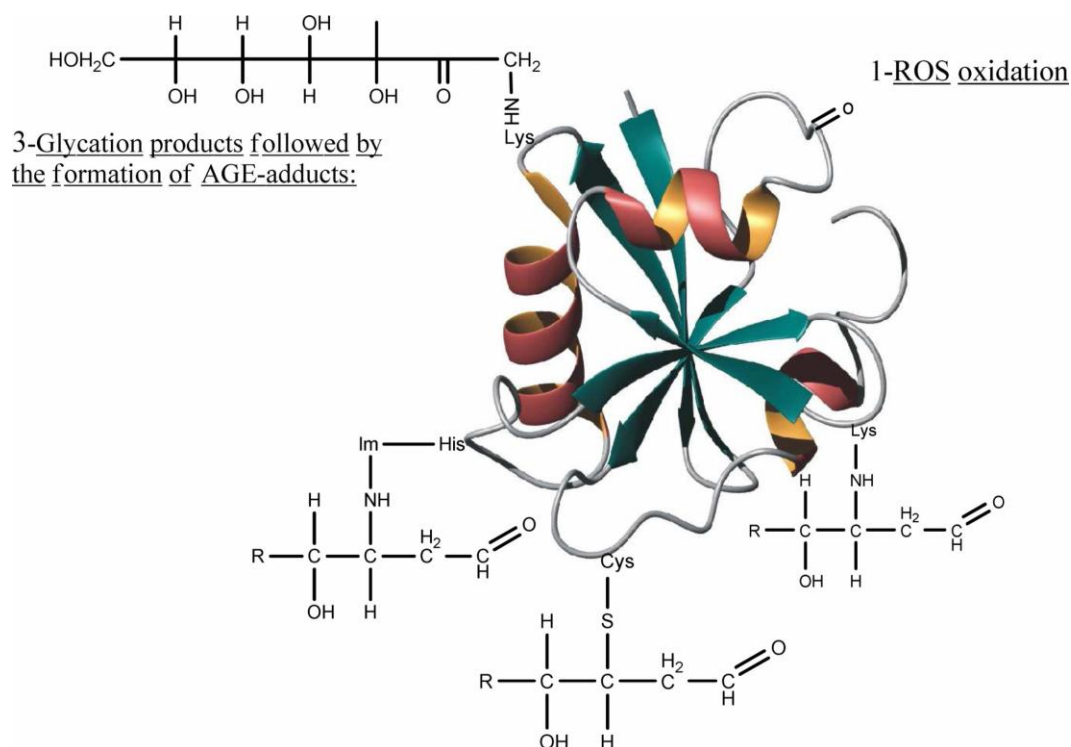
Although all amino acid residues of proteins are susceptible to oxidation, Cysteine (Cys) [Shacter 2000; Radi et al., 1991] and methionine (Met) [Shacter 2000, Vogt 1995] are those especially prone to oxidative attack, both of which contain susceptible sulfur containing groups (thiols). Under even mild conditions, in the case of cysteine residues, oxidation leads to the formation of sulfenic acid, inter- or intra-molecular disulfides, protein mixed disulfides with low molecular weight thiols (e.g., GSH), and S-nitrosothiols; methionine residues generate Met sulfoxide (MeSOX), the major product

of conversion under biological conditions. Most biological systems contain disulfide reductases and MeSOX reductases that can convert the oxidized forms of cysteine and methionine residues back to their unmodified forms. These reversible modifications can be part of regulatory processes of protein functions, in which cysteines can cycle between the oxidized and reduced state [Ahmed 2007].

However, Cys residues can be irreversibly oxidized by strong oxidative agents to sulfinic and sulfonic acids, which cause loss of protein function and cannot be reversed by metabolic processes [Woo et al., 2003]. In the case of prolonged storage, the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been found to undergo temporary change of its catalytic center in response to storage-induced oxidative stress through the formation of an intramolecular disulfide bond involving catalytic cysteines [Rinalducci et al. 2011].

1.3.2 Protein Carbonylation: Generation of Protein Carbonyl Derivatives

Protein carbonylation is considered a major form of protein oxidation and aging, not only because it is a sign of oxidative stress, but also of disease-derived protein dysfunction. Formation of protein carbonyls can occur either by the α -amidation pathway or by oxidation of glutamyl side chains which leads to formation of a peptide in which the N-terminal amino acid is blocked by α -ketoacyl derivative. However, direct oxidative attack on lysine, arginine, proline and threonine residues may also generate carbonyl derivatives [Berlett et al. 1997]. In addition, carbonyl groups may be introduced into proteins by Michael addition reactions with α,β -unsaturated aldehydes (4-hydroxy-2-nonenal, malondialdehyde) produced during lipid peroxidation [Schuenstein 1979, Esterbauer 1991, Uchida 1993] or with reactive carbonyl derivatives (ketoamines, ketoaldehydes, deoxyosones) generated as a consequence of glycation and glycoxidation reactions, in which reducing sugars or their oxidation products react with lysine residues of proteins [Monnier et al. 1995] (Figure 4).



2-Advanced lipid peroxidation end products:

Figure 4-A general scheme for the different routes for protein carbonylation [Ashraf Madian, 2010]

The higher levels of cytoskeletal protein carbonyl groups are observed after 35 days of prolonged storage of RBCs, especially in non-leukodepleted CPDA units, and this phenomenon is correlated with the extent of the oxidized Hb accumulation in the membrane and the cytoskeleton systematically after 10 days of storage [Annis et al., 2005; Kriebardis et al., 2007] (Figure 5). However, the observation of a decrease of cytoskeleton-bound Hb and protein carbonylation levels by the end of the storage period can be explained in part by the microvesiculation and the hemolysis of the most severely damaged cells, or by the proteasome activity, two recognized self-protective/age-dependent mechanisms [Kriebardis et al., 2006, Delobel et al., 2012]. Strictly related to the protein carbonyl production is the progressive accumulation of advanced glycation end-products, where glycated forms of haemoglobin (Hb1Ac) accumulate proportionally to storage duration. Notably, alterations to Hb1Ac levels in units that have been stored for longer periods might cause problems when treating/monitoring certain categories of patients, such as those suffering from diabetes. In parallel, altered membrane protein glycosylation patterns might influence the rheological properties of RBC.

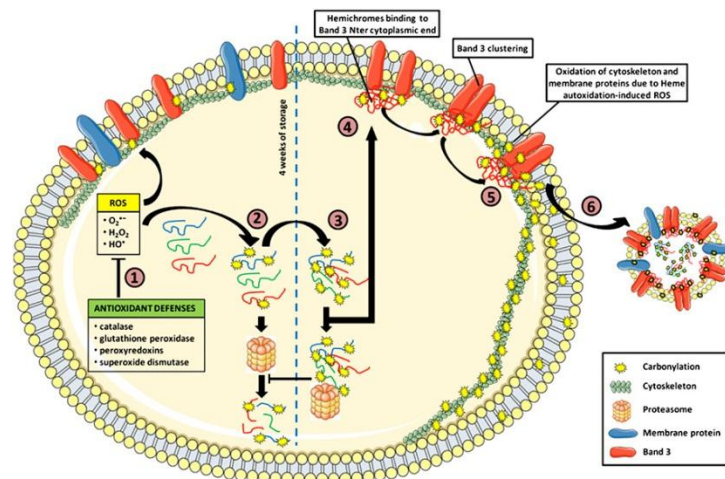


Figure 5-Carbonylation pathway in stored RBCs (1) RBC antioxidant defenses prevent protein carbonylation by reducing ROS into less reactive intermediates. After a certain storage period, these defenses are exceeded by the oxidative stress and protein carbonylation occurs on both soluble and membrane proteins. (2) Carbonylated proteins are recognized and degraded by the 20S proteasome. (3) Over-oxidized proteins crosslink and cannot be degraded by proteasome anymore, inhibiting degradation of fairly oxidized proteins as well as other damaged proteins. (4) Oxidized Hb aggregates as hemichromes and binds to and alters band 3 conformation. (5) Hemichromes heme autooxidation produces ROS which induces cytoskeleton and membrane protein carbonylation. (6) Membrane vesiculation allows elimination of altered band 3 and oxidized proteins [Delobel 2012].

1.3.3 Protein-Protein Cross-Linkage

Cross-linking is often seen in ROS induced protein oxidation, occurring in at least six different ways [Stadtman 1997, Berlett 1997, Mirzaei 2007]. Among the more frequent are a) formation of disulphide bonds between proteins through cysteine oxidation, b) Schiff base formation between a carbonyl group on an oxidized amino acid side chain of one protein and a lysine residue on another, c) Schiff base formation between the carbonyl group on an 4-hydroxynonenallysine (HNE) adduct of one protein and a lysine residue on another, d) the same process as with HNE but involving a malondialdehyde adduct on a protein, e) Schiff base formation again but with a carbonylated advanced glycation end products (AGE) product and another protein or f) by free radical cross-linking involving carbon centered radicals.

The red cell membrane skeleton is composed predominantly of four peripheral membrane proteins: spectrin, actin, protein 4.1, and ankyrin. Spectrin, the major red cell membrane protein, is a long, unusually flexible heterodimer that participates in the three major protein-protein interactions in the membrane skeleton [Shotton et al., 1979]: (i)

Spectrin associates with itself, head to head, to form tetramers [Shotton et al., 1979] and perhaps higher order oligomers [Morrow et al., 1981]; (ii) The opposite end of the molecule binds short filaments of actin, thus completing lateral connections within the skeleton; finally, (iii) this spectrin-actin association is greatly enhanced by the interaction of protein 4.1 with spectrin, near the spectrin-actin binding site. Several studies [Ohanian et al., 1984, Cohen et al., 1984, Platt et al., 1984] have demonstrated a spectrin injury (which interferes with spectrin-protein 4.1 interaction) remarkably similar to the storage lesion in a patient with hemoglobin Nottingham, that results from oxidation by breakdown products of stored red cells accumulated hemoglobin on the membrane. Moreover, oxidation of membrane proteins, due to treatment with 2mM periodate (known to oxidize vicinal hydroxyl groups in carbohydrates), results in a total cross-linking of the spectrin polypeptides [Gahmberg et al., 1978]. Moreover, during RBC storage, β -chain of hemoglobin becomes fused to the membrane fraction, mainly with intracellular portion of the membrane band 3 [Zhang et al., 2000], partially in a non-reducible, cross-linked form [Wolfe et al., 1986]. The formation of spectrin-Hb crosslinking represents a kind of widespread oxidative damage in RBC membrane that has been reported to normally occur in the more senescent cells *in vivo* [Snyder et al., 1983].

Another example emerges from studies about oxidative stress-dependent oligomeric status of erythrocyte peroxiredoxin II (PrxII) during storage under standard blood banking conditions [Rinalducci et al. 2011]. Its major functions comprise cellular protection against oxidative stress, modulation of intracellular signaling cascades that apply hydrogen peroxide as a second messenger molecule, and regulation of cell proliferation. In particular, human RBC PrxII, a 2-cys peroxiredoxin with a thiol-dependent H_2O_2 scavenger activity [Wood 2003], exhibits four different oligomeric states in cytosol, in which it exists as variable copies of its monomeric subunits, except that with the highest molecular weight (440kDa), containing both reduced and oxidized (disulphide-linked dimers) PrxII decamers. Although PrxII is a preferential cytosolic protein, its association to RBC membrane has been previously reported [Moore et al., 1991; Plishker et al., 1992; Moore et al., 1997; Murphy et al., 2004; Rocha et al., 2008; 2009; Antonelou et al., 2010]. In particular, the increase of intracellular reactive oxygen species, such as during storage, leads to the PrxII accumulation as dimer in the

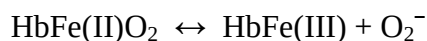
membrane; oligomeric complex at 440 kDa is converted to a higher molecular weight structure (480 kDa) due to the presence therein of cross-linked species of PrxII and hemoglobin, with the presence of both disulphide-linked PrxII dimers and monomer subunits, with a larger percentage of dimers.

Erythrocyte band 3 is a major multifunctional transmembrane glycoprotein divided into two rather different domains of similar size, the hydrophobic 52 kDa C-terminal domain and the water-soluble 43 kDa N-terminal domain (CDB3). The latter plays a crucial structural role in linking the bilayer with the spectrin-based skeletal network. Immunochemical data show immune recognition with the generation of aging-specific anti-band 3 neoantigens in the oldest erythrocytes in concomitant with an apparent aging associated increase in aggregation of band 3 [Kriebardis et al., 2007].

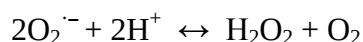
1.3.4 Hemoglobin oxidation

Hemoglobin, being the most abundant (98% of total protein content) protein in RBC, is the main generator of reactive oxygen species, the main target of oxidative damage and also a scavenger of free radicals.

In this way, the binding of oxygen to Hb, often denoted in the literature as oxygenated Hb (oxyHb) or HbFe(II)O_2 , is accompanied by a migration of charge from the heme iron to the oxygen, such that the structure of oxyHb is a ferric-superoxide anion complex ($\text{Fe}^{3+}\text{O}_2^-$) [Misra et al., 1972] (Figure 6). Oxy-Hb is a fairly stable molecule but does slowly auto-oxidize to metHb. The autoxidation of oxyHb involves the dissociation of the oxygen without electron transfer to yield superoxide (O_2^-) and metHb [HbFe(III)] [Misra et al., 1972].



Hydrogen peroxide (H_2O_2) is produced during Hb autoxidation by the spontaneous and enzyme driven dismutation of superoxide.



Under normal unstressed conditions, the red cell reducing capacity is greater than 250 times its oxidizing potential: metHb is normally reduced back to deoxygenated Hb (deoxyHb) by NADH–cytochrome b5–metHb reductase [Moore et al., 2004] and the superoxide is dismutated without consequences. However, MetHb can react with hydrogen peroxide to generate two oxidizing species: oxoferrylHb and a protein radical centered on the globin chain [Winterbourn 1985, Sztiller et al., 2006]. This short-lived ferryl radical reacts rapidly with oxygen to yield peroxy radicals, which can degrade to irreversibly green Hb derivatives including choleglobin and sulfhemoglobin [Winterbourn 1985, Moxness et al., 1996]. Both derivatives are similar in having covalently modified porphyrin rings and a hydrophobic affinity that enables them to bind lipids. This feature has been linked to membrane damage through the extraction of membrane components, such as phosphatidylserine, leading to shape change and phospholipid redistribution [Moxness et al., 1996, Brunauer et al., 1994].

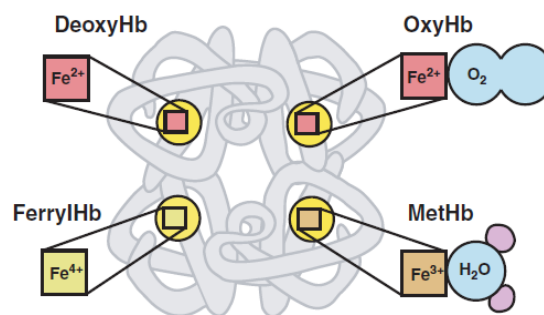


Figure 6—Different oxidative states of the heme iron. Each of the four globin chains of the hemoglobin (Hb) molecule contains a heme group with an iron atom at its center. Hb oxidation can involve changes in the iron's oxidative state, and, consequently, modifications in ligand binding. The reduced ferrous state (Fe^{2+} , red) allows oxygen binding to deoxygenated Hb (deoxyHb) at physiological conditions. The resultant oxygenated Hb (oxyHb) can undergo oxidation to methemoglobin (metHb), in which the heme iron is in the ferric state (Fe^{3+} , brown). MetHb can bind water, but not oxygen under physiological conditions. The ferryl state (Fe^{4+} , green) can be obtained during Hb oxidative injury by hydrogen peroxide. This state can lead to Hb denaturation [Misra et al., 1972].

Nevertheless, several erythrocyte abnormalities that circumvent or overwhelm the erythrocyte oxidant defense system have been identified, such for example, those related to hypothermic storage. In fact, although cooling below normal physiological temperatures partially contrasts the accumulation of oxidative injuries, (I) the rate of met-Hb reduction by cytochrome b5 reductase is slowed; (II) met-Hb may be more

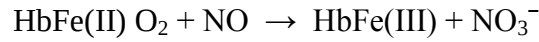
prone to denaturation as suggested by lower thermodynamic stability of met-myoglobin at 4°C; and (III) the solubility of oxygen is doubled at 4°C. As a result, oxidative damage can accumulate with refrigerated red cell liquid storage [Zolla and D'Alessandro, 2011]. Moreover, the storage means (e.g. plastic bag and additive solutions) contribute to exacerbate this phenomenon, by promoting an increased concentrations of intracellular ROS during blood storage [Deepa Devi et al., 1998].

Under certain conditions, including heat stress, changes in pH, senescence [Sugawara et al., 2003] and also increased Hb autoxidation, MetHb can be converted into low-spin ferric Hb compounds named hemichromes (haemoglobin whose cysteine residues have been oxidised, leading to the formation of aggregates). The release of the heme moiety by denatured hemichromes has been shown to initiate oxidative damage to adjacent membrane components [Atamna et al., 1995, Li et al., 2006]. Free heme has been shown to inhibit a number of cytosolic enzymes [Scott et al., 1993], as well as to react nonspecifically with membrane proteins leading to abnormal thiol oxidation and cross-linking [Atamna et al., 1995] (especially at the level of the cytoplasmatic domain of band 3), with consequent reduction of its deformability. Although free heme is degraded *in vivo* through the heme oxygenase system [Kapitulnik 2004], during prolonged storage, when the flux of glycolytic pathway decreases and NADH concentrations fall, release of the heme iron has been shown to be catalyzed by intracellular GSH [Atamna et al., 1995] or hydrogen peroxide [Nagababu et al., 1998]. As previously stated, free iron is potentially toxic capable of acting as a Fenton reagent in the Haber–Weiss cycle, which produces the deleterious hydroxyl radicals (OH•) and finally results in the formation of Heinz bodies [Chiu et al., 1989] (Figure 7).

1.3.5 Hemoglobin and the functions of nitric oxide (NO) correlated with the Red Cell Storage Lesion

Although hemoglobin can induce significant oxidative stress and inflammation, a central mechanism for toxicity is likely the direct effect of free hemoglobin on nitric oxide (NO) catabolism. Nitric oxide has a myriad of functions in human physiology including neurotransmitter, host-defense molecule, inhibitor of platelet aggregation, regulator of endothelial adhesion molecule expression, and antioxidant. It is perhaps most widely recognized for its role in metHb formation. In fact, during this reaction,

that is rate limited by diffusion to the heme group within Hb [Doyle et al., 1981, Eich et al., 1996, Herold et al., 2001], oxygenated Hb reacts with NO to form metHb (FeIII) and nitrate (NO_3^-), and effectively destroys NO activity.



This rapid binding means a reduced NO bioavailability for vasodilation, although the erythrocyte can be considered a potent NO sink [Kevil et al., 2010, Ferrari et al., 2009]. However, as noted above, cell-free Hb reacts with NO much faster (1000 times) than RBC-encapsulated Hb. With increasing length of storage, the concentration of free hemoglobin (in the form of ferrous oxyhemoglobin) in RBCs increases, with a direct, proportional consumption of NO to the concentration of free heme. In addition, after a dramatic increase in the number of microparticles in stored RBCs, cell-free Hb and Hb-containing microparticles scavenge NO very fast. It is easy to see how disruption of the RBC membrane, as in hemolysis (with a range of extracellular Hb levels reported in the literature from 28 mmol/L - in heme - after 35 days of storage to 80 mmol/L after 50 days of storage [Bonaventura et al., 1997]), will effectively contribute to affect NO bioavailability upon transfusion. Nevertheless, several mechanisms exist to prevent the depletion of NO through interaction with hemoglobin. The first is that hemoglobin located within the RBC membrane (RBC-encapsulated Hb) reacts with NO much more slowly (1000 times) than does cell-free hemoglobin [Coin et al., 1979], primarily due to the limitation of NO diffusion to erythrocyte through “an unstirred layer” [Azarov et al., 2005]. A cell-free zone at the blood-endothelial interface is created by laminar blood flow, which pushes RBCs to the center of the vessel and away from endothelial cell, where NO is produced. In addition, the RBC membrane has a finite permeability to NO. Indeed, any NO at greater distances from the external surface of the red cell membrane has the rate limited by NO diffusion to the red cell. It is of great biological significance that the Stamler laboratory has demonstrated another relevant reaction of NO with Hb: S-nitrosylation of a cysteine residue, conserved in β -globins of all birds and mammals (βCys93 of human Hb), which yields S-nitrosohemoglobin (SNO-Hb) [Jia et al., 1996, Gow et al., 1998, Bonaventura et al., 1997].

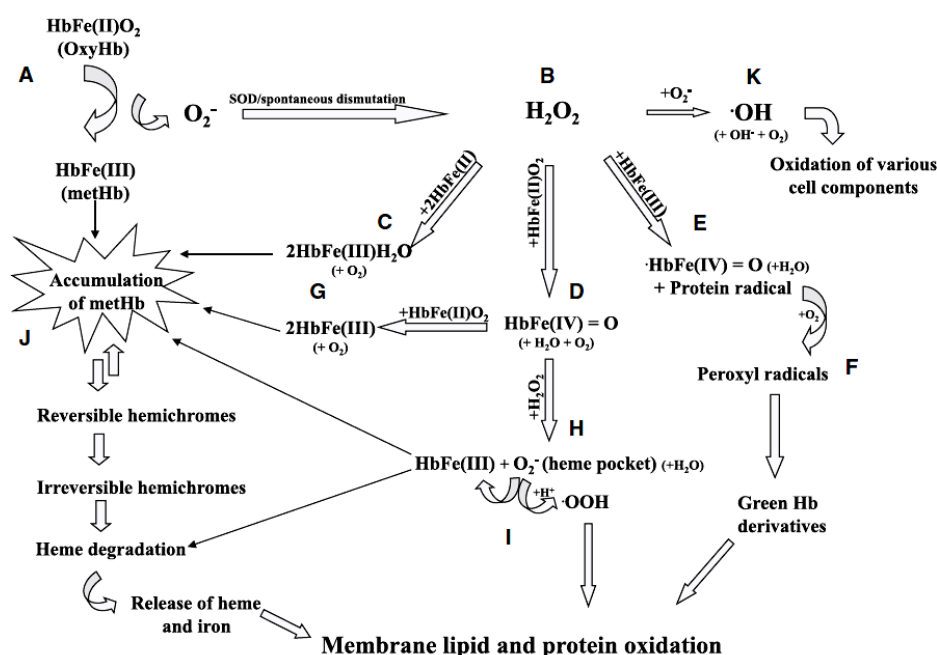


Figure 7—Summary of the major hemoglobin (Hb) oxidative pathways and their link to membrane damage. (A) Autooxidation of oxygenated Hb [oxyHb, HbFe(II)O₂] generates methemoglobin [metHb, HbFe(III)] and superoxide anions (O₂^{•−}). Spontaneous or enzymatic dismutation of O₂^{•−} generates hydrogen peroxide (H₂O₂). (B) Accumulation of H₂O₂ caused by the loss of vital antioxidants (catalase, glutathione) may cause irreversible oxidative damage to Hb molecules. H₂O₂ can react with deoxygenated Hb [HbFe(II)], oxyHb and metHb to generate metHb (C), ferrylHb [HbFe(IV)=O] (D) and oxoferrylHb [HbFe(IV)=O] (E), respectively. The reaction between H₂O₂ and metHb (E) also generates a protein radical that can further react with oxygen to yield peroxyl radicals (F) and, eventually, decompose to lipophilic green Hb derivatives, which bind to membrane lipids and induce oxidative damage. FerrylHb (D) can react with oxyHb to generate two molecules of metHb (G), or with H₂O₂ (H) to generate metHb and O₂^{•−}. The formation of O₂^{•−} within the heme pocket of the Hb has been shown to result in heme degradation. Alternatively, a protonation of O₂^{•−} generates perhydroxyl radicals (•OOH) (I) that are known to initiate membrane lipid peroxidation. Accumulation of intracellular metHb (J) may result in the formation of hemichromes. Irreversible hemichromes undergo further denaturation leading to heme degradation and release of the heme moiety and iron atoms. Both degradation products have been shown to initiate membrane oxidative damage. The presence of O₂^{•−} and H₂O₂ may result in the formation of hydroxyl radicals (•OH) (K) through the Haber–Weiss reaction. Heme degradation releases iron atoms, which can catalyse this reaction. •OH is highly reactive and can cause oxidative damage to various cell components. Note that all three major oxygen radicals (O₂^{•−}, H₂O₂ and •OH) can take place in the oxidative denaturation of Hb and membrane damage. Remark: In order to simplify this model, reaction products that do not participate in oxidative pathways (i.e. H₂O, O₂) are denoted by smaller fonts in parentheses [Kanas et al., 2009].

Importantly, under hypoxic conditions, SNO-Hb retains endothelium-derived relaxing factor (EDRF/NO) like bioactivity and is capable of transferring NO to low molecular weight thiol-containing molecules (e.g. the membrane anion exchange protein, glutathione). Notably, allosteric structural transitions of Hb, triggered by changes in oxygen tension in vivo, contribute to the molecular gymnastics that dictate NO addition and release from Hb-βCys93 [Jia et al., 1996, Gow et al., 1998, Bonaventura et al., 1997].

Although mechanistic details continue to be debated, recently it has been observed that levels of SNO-Hb are altered in several disease states, characterized by disorders in tissue oxygenation [Reynolds et al., 2007], like these affecting banked blood. The storage-related deficiency of SNO-Hb, with associated impaired vasoregulation hinders to improve oxygen delivery by RBCs in the microcirculation, predisposing excess mortality and morbidity accompanying RBC transfusion. However, under conditions of exacerbated Hb autoxidation (like *ex-vivo* storage of RBCs), when produced in excess, a significant amount of $O_2^{\cdot-}$ reacts with NO to produce peroxynitrite ($ONOO^-$), a strong oxidant formed intravascularly [Darley-Usmar et al., 1995], with a very short half-life (g.e. milliseconds). Peroxynitrite can promote the nitration of aromatic compounds such as protein tyrosine residues, a process that can profoundly alter protein structure and function, and requires the intermediate formation of secondary nitrating species. Generally, peroxynitrite can behave as either a one- or two-electron oxidant; in this particular case, one-electron oxidants derived from peroxynitrite attack the phenolic ring, leading to formation of a tyrosyl radical, followed by a coupling of the amino acid-derived radical with $\cdot NO_2$ to yield 3-nitrotyrosine (3-NT) [Romero et al., 2003]. The biological significance of tyrosine nitration supports the formation of 3-NT *in vivo* in diverse pathologic conditions and the formation of 3-NT is thought to be both a relatively specific marker of oxidative damage mediated by peroxynitrite and other nitrogen free radical species [Radi 2013] and a post-translational modification with important pathophysiological consequences, that disrupts NO signaling and skews metabolism towards pro-oxidant processes. Concerning RBCs, recent studies have shown that peroxynitrite induces morphological alterations, phosphatidylserine externalization, activation of caspase cascades, increased amount of membrane-bound Hb (presumably oxidized/denatured Hb), band 3 phosphorylation and a burst of both the glycolytic pathway (with lactate accumulation) and the pentose phosphate shunt [Matarrese et al. 2005, Metere et al., 2009]. Interestingly, all of these are also features of storage lesions, thus the investigation of protein nitration in stored RBCs would be beneficial and of great interest.

1.3.6 Lipid Peroxidation

It is generally believed that membrane lipids are major targets for cellular damage induced by free radicals. Red blood cells offer a number of advantages studies of effects of oxidants on protein and lipid breakdown and oxidation respectively. As previously stated, oxygen radicals are produced continually in red cells and may cause and damage macromolecules and cellular structure of the organism, endothelium erythrocytes and lipid peroxidation (the level of lipid peroxidation expressed as malondialdehyde). The latter initiates when ROS remove a hydrogen atom from methylene carbons of fatty acid side chains of Polyunsaturated fatty acids (PUFAs), resulting in lipid radical which reacts with molecular oxygen to yield peroxy radicals. Peroxy radicals propagate the oxidative process by removing hydrogen atoms from adjacent fatty acids, and consequentially create various lipid hydroperoxides which further decompose to secondary lipid peroxidation products such as malondialdehydes (MDAs) [Boaz et al., 1999; Fiorillo et al., 1998]. MDAs have been widely used as lipid peroxidation indicators. MDAs have been shown to have adverse effects on cell integrity as shown by membrane damage and hemolysis in RBCs. The lipid peroxidation of polyunsaturated fatty acids may be enzymatic and non-enzymatic. Enzymatic lipid peroxidation is catalyzed by the lipoxygenases family, a family of lipid peroxidation enzymes that oxygenates free and esterified PUFA generating as a consequence, peroxy radicals. Researchers have focused their attention on lipid peroxidation by non-enzymatic, non-radical mechanisms and formation of lipidperoxides, which is initiated by the presence of molecular oxygen and is facilitated by Fe^{2+} ions [Repetto et al., 2010]. Singlet oxygen and ozone are examples of molecules that induce such oxidation. The presence of cholesterol in cell surface membranes influences their susceptibility to peroxidation, probably both by intercepting some of the radicals present and by affecting the internal structure of the membrane by interaction of its large hydrophobic ring structure with fatty acid-side-chains. As lipid peroxidation proceeds in any membrane and several of the products, including hydroperoxides, cleavage products such as aldehydes, and polymeric materials produced, have a detergent-like activity, or exert cytotoxic and genotoxic effects. This will contribute to increased membrane disruption and further peroxidation.

The onset of lipid peroxidation within biological membranes is associated with changes in their physicochemical properties and with alteration of biological function of lipids and proteins [Catala 2006].

1.3.7 The effects of phosphorylation on integrity membrane structure of stored RBCs under blood banking conditions

It has been known for decades that phosphorylation plays an important role in cell cycle, apoptosis, metabolism, receptor function and stress responses, through the activation of signaling cascades [Dephoure et al., 2008, Macek et al., 2009, Beltran et al., 2012]. The identification of the storage-associated alterations in the interaction between the cytoskeleton and the lipid bilayer that determine membrane elasticity has been widely documented [Berezina et al., 2002, Blasi et al., 2012, Cluitmans et al., 2012]. Protein phosphorylation/dephosphorylation is one of the physiological processes possibly controlling the membrane stability. In fact, recent data indicate that phosphorylation is involved in molecular interactions at several levels and thereby contributes to the integrity of the general membrane structure [Manno et al., 2005, De Oliveira et al., 2008]. In particular, a central position on the functionally relevant changes in the membrane composition of stored RBCs is occupied by alterations of phosphorylation status of band 3. Cluitmans and colleagues have postulated that altered phosphorylation of band 3 on tyrosine residues, due to oxidative stress, may be responsible for the metabolic changes, such as the described increase in glycolytic intermediates within the first two weeks of storage [D'Alessandro et al., 2012, Gevi et al., 2012] (in fact, the cytoplasmic domain of band 3 has a high affinity for key enzymes of the glycolysis, and binding is regulated by phosphorylation). The oxidative stress-dependent increase in band 3 tyr-phosphorylation is a trigger for activation of src tyrosine kinases (Syk and Lyn) [Mallozzi et al., 2001] and down-regulation of tyrosine phosphatases (PTP1B, SHP-1 and SHP-2) [Zipser et al., 2002]. The src kinases phosphorylate tyrosines 8 and 21 of the cytosolic domain of the band 3 protein, thereby facilitating formation of high-molecular-mass band 3 aggregates [Pantaleo et al., 2009]. Both srk kinases syk and lyn and the tyrosine phosphatase PTP1B are redox-sensitive, indirectly the first, because their activity is controlled by the redox- and calcium-sensitive phosphorylation steps mediated in particular by protein kinase C alpha. On the

contrary, PTP1B possesses cysteine residues in the kinase domains, whose oxidation is direct induced by oxidative stress [Bordin et al., 2005, Knock and Ward, 2011]. The resulting cumulative tyrosine hyperphosphorylation, during last weeks of storage, decreases the affinity of band 3 to ankyrin, causing destabilization of the band 3-cytoskeleton interaction, increases the lateral mobility of band 3 within the membrane and induces a progressive vesiculation [Ferru et al., 2011]. In addition, recent researches have demonstrated the association between casein kinase II-catalyzed phosphorylation of beta spectrin serine and protein kinase C- catalyzed phosphorylation of protein 4.1 and complement receptor- mediated increase in deformability [Glodek et al., 2010]. Phosphorylation of band 4.1 promotes dissociation of actin from the cytoskeleton, also contributing to an increase in deformability [Manno et al., 2005, De Oliveira et al., 2008].

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CHAPTER 2:
HAEMOGLOBIN GLYCATION (Hb1Ac) INCREASES DURING RED BLOOD
CELL STORAGE: A MALDI-TOF MASS-SPECTROMETRY-BASED
INVESTIGATION

2.1 AIM OF THE STUDY

Haemoglobin A1c (HbA1c) represents a key biomarker in diabetes diagnosis and management, since it allows clinicians to estimate mean blood glucose concentration in the recent period preceding withdrawal [Stevens et al., 1977]. Indeed, glycation of haemoglobin is a non-enzymatic irreversible process that is promoted by the prolonged exposure of erythrocytes to high glucose concentrations [Stevens et al., 1977], a condition that is known to occur under blood banking conditions, where additive solutions (such as SAGM) expose red blood cells (RBCs) to higher than normal glycemic levels [Szelényi et al., 1983]. However, controversial data indicate no clear hint as to whether and to which extent HbA1c accumulates during red blood cell storage [Szelényi et al., 1983, Weinblatt et al., 1986, Spencer et al., 2011]. Indeed, data from the older literature support the hypothesis that high glucose concentrations in RBC storage medium end up promoting glycation of Hb, thus resulting in the accumulation of HbA1c over time [Szelényi et al., 1983, Weinblatt et al., 1986]. These results would underpin the prediction about HbA1c increasing in transfused recipients, although no statistically significant correlation between these two events has been observed by Spencer and colleagues, who thus concluded that ‘glycation of haemoglobin in stored RBC units is negligible despite the high glucose concentrations in stored RBC units’ [Spencer et al., 2011]. Hereby, we propose the application of a validated [Biroccio et al., 2005, Zurbriggen et al., 2005, Bursell et al., 2000] MALDI-TOF mass-spectrometry-based method to this issue and report the observation about HbA1c levels apparently increasing over storage progression.

2.2 MATERIALS AND METHODS

2.2.1 Sample collection

RBC units were drawn from healthy human volunteers according to the policy of the Italian National Blood Centre guidelines (Blood Transfusion Service for donated blood) and all the volunteers provided their informed consent in accordance with the declaration of Helsinki. We studied RBC units collected from 10 healthy donor volunteers [male = 5, female = 5, age 42.3 ± 10.5 (mean $\pm$ S.D.)]. In details, whole blood (450 mL + 10%) was collected from healthy volunteer donors into CPD

anticoagulant (63 mL) and leukodepleted (i.e. 4-log WBC depletion). After separation of plasma by centrifugation, RBCs were suspended in 100 mL of SAGM (Saline, Adenine, Glucose, Mannitol) additive solution. RBC units were stored for up to 42 days under standard conditions (1–6°C), while samples were removed aseptically for the analysis fortnightly (at 0, 14, 28 and 42 days of storage).

2.2.2 Matrix and sample preparation for MALDI-TOF MS

Protein calibration standard mix I (containing horse heart cytochrome c and myoglobin in the HbA1c target molecular weight range) was obtained from Bruker Daltonics (Bremen, Germany). Sinapinic acid (3,5-dimethoxy-4-hydroxy-cinnamic acid - SA), solvents for sample preparation and trifluoroacetic acid (TFA) were purchased from Fluka (Milan, Italy).

MALDI-TOF MS analysis of HbA was performed according to the method by Biroccio et al. [Biroccio et al., 2005]. Samples were prepared with a sandwich layer method. First, a matrix layer was prepared by applying a droplet (0.5 µL) of 30 mg/mL of saturated solution of SA matrix dissolved in 100% ethanol to the surface of a smooth stainless steel sample plate and allowing it to dry. A fine crystalline layer was formed within a few seconds. A droplet (0.5 µL) of 30 mg/mL of saturated solution of sinapinic acid matrix, dissolved in acetonitrile and 0.1% trifluoroacetic acid (1:1), and analyte solution (1:100 dilution), mixed with equal volumes (5 µL), was placed on the surface of the crystalline seed layer and dried in air at room temperature. The stainless steel sample plate (MTP384 target plate ground steel – Bruker Daltonics - Bremen, Germany) was then inserted into the MALDI instrument.

2.2.3 Linear MALDI-TOF MS analysis and calculation of glycated or glutathionylated hemoglobin

All MALDI analyses were performed with an Autoflex III MALDI-TOF mass spectrometer (Bruker Daltonics - Bremen, Germany). The system utilizes a Smartbeam laser, emitting at 337 nm. Ion source and flight tube were kept at a pressure of about 4×10^{-7} mbar by turbo molecular pumps. All spectra were acquired in linear mode at 20, 18.5, and 6.80 kV for ion sources 1 and 2 and lens, respectively. Laser power was fixed to 25% with a pulsing rate of 200 Hz to allow about 1000 ion counts for each single

acquisition series (500 shots). Spectra analysis was performed with Bruker FlexAnalysis 3.3 (Bruker Daltonics – Bremen, Germany). Relative concentrations of HbA1c were calculated as a percentage of the glycosylated forms (15.289 and 16.030 m/z) with respect to the total of the free form of alpha and beta globin chains (Hb= 15.127 +15.868 m/z), within the linearity range of the instrument [Stevens et al., 1977], according to the following equation:

$$\text{HbA1c \%} = \frac{\text{HbA1c}}{(\alpha + \text{HbA1c})} \times 100$$

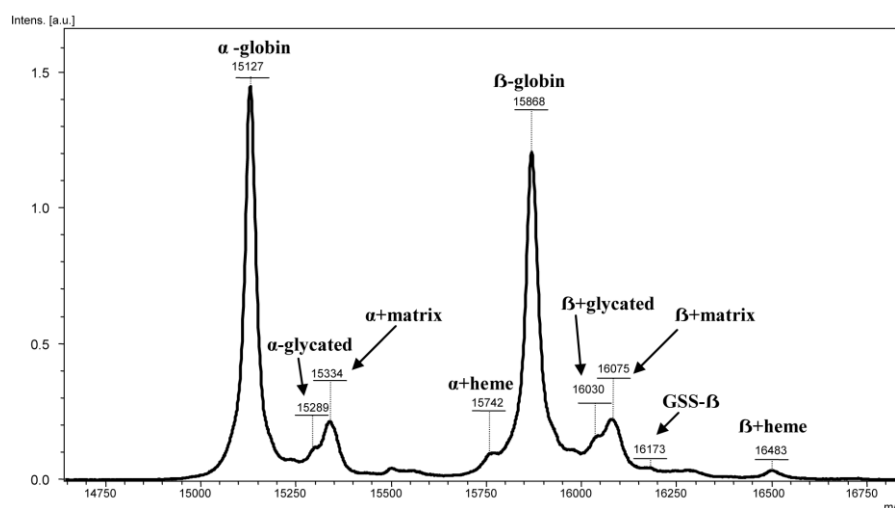


Figure 1-MALDI TOF intact mass spectrum of alpha and beta globin chains. Spectrum interpretation and peak attribution has been performed on the basis of Biroccio et al. and Zurbriggen et al. A sample RBC intact mass spectrum is reported in the range between 14500 and 17000 m/z. We could identify the main alpha (15127 m/z) and beta globin (15868 m/z) chains, their main glycosylated variants showing a +162 Da adduct (at 15 289 and 16 030 m/z, respectively), and the respective heme adducts (+615 Da, at 15 742 and 16 483 m/z, respectively) [Biroccio et al., 2005, Zurbriggen et al., 2005]. Also, +207 Da adducts with the SA matrix were visible as well, in agreement with Biroccio et al.

3 RESULTS AND DISCUSSION

The analytical approach exploited in the present study holds several advantages over routine methods for HbA1C, including the rapidity and robustness of the approach, along with the higher sensitivity of MALDI-TOF-TOF last generation instruments in comparison with triple quadrupoles mass spectrometers. Triple quadrupoles are more diffused in the clinical setting [Gevi et al., 2012], despite their low tolerance for salts

and sample impurities, instrumentation costs, length of the analysis and laborious sample handling/preparation [Gevi et al., 2012]. In Figure 1, we report a sample MALDI-TOF spectrum. In Figure 2, where spectra from the analysis of RBCs at 0, 14, 28 and 42 days of storage are graphed, storage progression corresponded to a visible increase in the glycated forms of Hb chains. Results reported in Table 1 are representative of the quantification of the relative percentages of HbA1c, according to the formula indicated in Table 1. As it emerges from these results, relative quantities of glycated Hb form A1c in relation to free Hb alpha chain increase over storage progression, as to become statistically significant (P-value < 0.05 ANOVA) after the 14th day of storage, at 28 days. Therefore, our results are consistent with older direct measurements of HbA1c in stored RBC units [Szelényi et al., 1983, Weinblatt et al., 1986]. Although these results are in apparent contradiction with the report by Spencer and colleagues [Spencer et al., 2011], it should be noted that indirect measurements of HbA1c in the recipients upon transfusion are both affected by pretransfusion HbA1c levels and dilution of the HbA1c levels of long-stored transfused unit (in a healthy patient, this ratio is approximately 1:10 for a single RBC unit in a 5-l adult male healthy individual blood sample). Also, the final HbA1c upon long-term storage might indeed increase over initial values (of the donor), but still be lower than the one that could be observed in the pre-transfusion recipient's blood. Accumulation of HbA1c might be dependent on the additive solution. Indeed, absolute and relative quantifications of glucose levels in CPD-SAGM-stored erythrocyte concentrates indicate that, despite ongoing glycolysis [Gevi et al., 2012, D'Alessandro et al., 2012], at the end of the storage, glucose levels in the supernatants are approximately of 12 – 1 mM, which is still higher than circulating glucose in diabetic patients (subjects with a consistent glycaemia above 7 mM are generally held to have diabetes) [Burger et al., 2010]. If we consider that HbA1c formation is a non-enzymatic phenomenon that is both dependent on glucose concentrations and oxidative stress, one leading cause triggering RBC storage lesions during prolonged storage [Gevi et al., 2012, D'Alessandro et al., 2012], it is realistic enough to conclude that CPD-SAGM-stored RBCs should be more susceptible to the formation of HbA1c than RBCs storage in presence of other, less 'glucose loaded', additive solutions. In this view, it is worthwhile to stress that oxidative stress in CPD-SAGM-stored RBC units becomes significant upon 2 weeks of

storage [Gevi et al., 2012, D'Alessandro et al., 2012], in line with the hereby reported observations (Table 1).

Table 1 – Time course analysis of HbA_{1c} during red blood cell storage	
Sample	Mean Value $\text{HbA}_{1c} \% = \frac{\text{HbA}_{1c} \times 100}{(\text{Hb} + \text{HbA}_{1c})}$
0-day	4,13 + 0.25
14-day	5,36 + 0.18
28-day	7,59 + 0.21*
42-day	10,90 + 0.25*

Hb: ion counts at 15127 m/z + 15868; HbA_{1c}: ion counts at 15289 + 16030 m/z
 * = statistically significant at $p < 0.05$ ANOVA

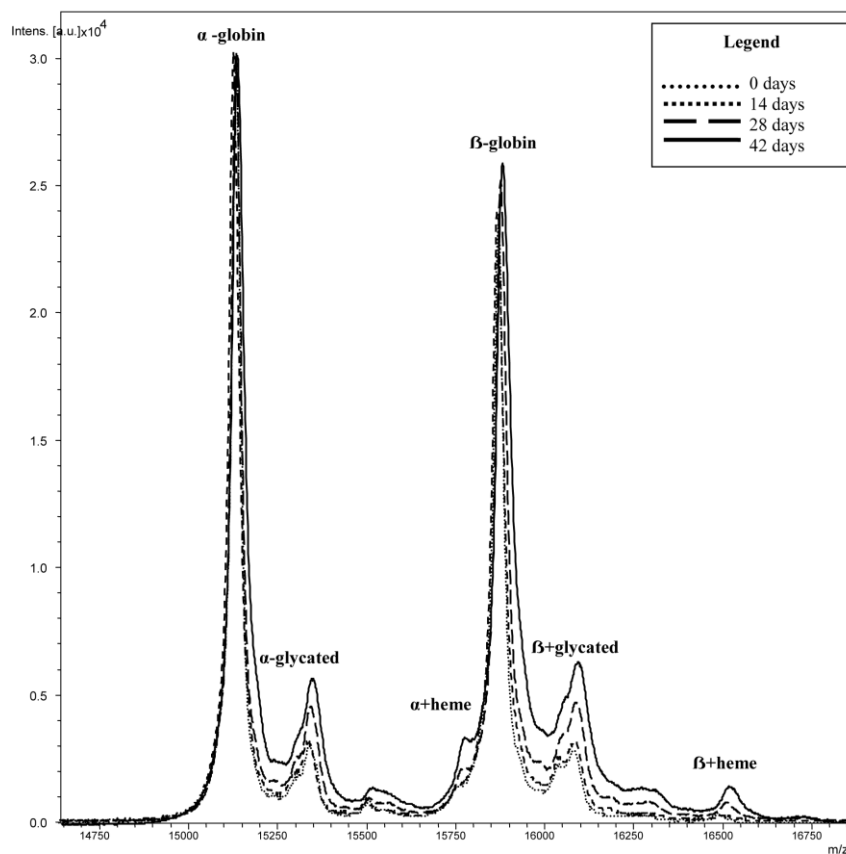


Figure 2-Time course analysis of RBC extracts over storage duration under blood bank conditions. Samples were assayed fortnightly at 0, 14, 28 and 42 days of storage. HbA_{1c} glycated form of alpha globin evidently increased in proportion to storage duration. A small peak was also visible (especially in long stored RBC samples) at 16 173 m/z, corresponding to the +305 Da glutathionylated adduct of the Hb beta chain, in agreement with [Bursell et al., 2000].

4 CONCLUSION

Although recent evidences suggest that storage duration ends up influencing HbA1c measurements in transfused recipients only to a negligible extent [Spencer et al., 2011], we hereby confirm old literature data [Szelényi et al., 1983, Weinblatt et al., 1986] through alternative, yet validated, MALDI-TOF mass-spectrometry-based approaches that the relative quantities of glycated HbA1c increase over storage duration.

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CHAPTER 3:
RED BLOOD CELL METABOLISM UNDER PROLONGED
ANAEROBIC STORAGE

3.1 AIM OF THE STUDY

The main biological function of red blood cells (RBCs) is to deliver molecular oxygen (O_2) to tissues, a task that they fulfill through the allosteric regulation of hemoglobin (Hb) [Monod et al., 1965]. Allosteric regulation is based upon the transition from a T-state to a relaxed R-state and vice versa, the former displaying lower oxygen affinity that results in oxygen release at the tissue level. The T state of Hb (deoxygenated Hb) is stabilized by several factors, including low pH, high CO_2 and organic phosphates, including 2,3-diphosphoglycerate (DPG) and ATP [Jensen et al., 2004]. Accelerated glucose consumption, leading to lactate accumulation through the Embden–Meyerhof glycolytic pathway, fuels the generation of DPG and ATP to stabilize the system under hypoxic conditions, [Murphy et al., 1960, Hamasaki, et al., 1970] in a Pasteur effect-like phenomenon [Marshall et al., 1977]. However, despite a 26% increase in the glycolytic rate upon deoxygenation of RBCs, Rapoport et al. reported that adenine nucleotides and DPG remained constant, as they become increasingly bound to deoxy-Hb [Rapoport et al., 1966].

The oxygenation state of Hb and metabolism are further intertwined, through the indirect modulation of glycolytic enzyme activities via competitive binding to the cytoplasmic domain of band 3 (CDB3) [Low et al., 1986, Chu et al., 2008]. Indeed, deoxy-Hb displays a higher affinity for the CDB3 than does oxy-Hb [Walder et al., 1984, Che'rite and Cassoly et al., 1985]. The CDB3 is a membrane docking site for several enzymes of the glycolytic pathway, such as phosphofructokinase (PFK), aldolase, glyceraldehyde- 3-phosphate dehydrogenase (GAPDH) and lactate dehydrogenase (LDH) [Low et al., 1986, Chu et al., 2008]. When bound to the CDB3 these enzymes are inhibited, while their displacement from the membrane in consequence to the binding of deoxy-Hb to the CDB3 results in their activities being restored and thus, in metabolic modulation [Lewis et al., 2009]. Several models have been proposed over the years, out of which the most widely accepted one is the one described in detail by Low's [Low et al., 1986, Chu et al., 2008, Lewis, et al., 2009] and Giardina's [Castagnola et al., 2010, Messina et al., 1996, Galtieri et al., 2002] groups, both stressing the role of oxygenlinked modulation of erythrocyte metabolism. More recently, a role for the phosphorylation state of the tyrosine residues of the band 3

protein at positions 8 and 21 in modulating the binding of glycolytic enzymes and deoxy-Hb to the CDB3 has been indicated [Lewis et al., 2009]. Phosphorylation to these residues results in an increased (+45%) glycolytic flux and reduced shift towards the pentose phosphate pathway (PPP) (-66%) than in oxygenated RBCs [Lewis et al., 2009]. The underlying mechanism seems to involve the phosphorylation-dependent increase in the number of negative charges at the N-terminal domain of band 3, which greatly enhances Hb binding to band 3 and thus the displacement of glycolytic enzymes [Lewis et al., 2009]. Notably, deoxygenation seems to promote phosphorylation of the CDB3 [Siciliano et al., 2005]. Over the years, several groups have contributed substantial efforts to the continuous improvement of existing mathematical and in silico models of RBC metabolism [Moses et al., 1972, Wiback and Palsson, 2002, Jamshidi, et al., 2001, Mulquiney, et al., 1999, Holzhutter, et al., 1985, Schauer, et al., 1981, Ataullakhanov et al., 1981, Heinrich, et al., 1977, Werner and Heinrich, 1985, Nakayama et al., 2005] towards the achievement of a Systems Biology understanding of RBC metabolic complexity [Jamshidi and Palsson, 2006]. In this view, Kinoshita and colleagues recently investigated the role of allostery in hypoxia-induced immediate metabolic alterations (within a 3 minutes framework upon deoxygenation) in RBCs through in silico prediction models (E-Cell 3 simulation environment) and experimental testing by means of capillary electrophoresis coupled with mass spectrometry (MS) [Kinoshita et al., 2007].

Over the last few decades, several groups have monitored RBC metabolic fluxes under control (aerobic) conditions during a prolonged time span, while exploiting international guidelines for optimized RBC concentrate storage conditions as a model [Messana et al., 1999, Bennett-Guerrero et al., 2007, Messana et al., 2000, Gevi et al., 2012, Nishino et al., 2009]. From these studies it emerged that, under control storage conditions (storage in the presence of additive solutions such as SAGM [Gevi et al., 2012] and PAGGM, [Nishino et al., 2009] under refrigeration at 1–6 °C for up to 42 days) RBCs face a rapid fall of the glycolytic rate and undergo an accumulation of glycolysis end products. A shift was observed towards the oxidative phase of PPP, [Gevi et al., 2012] in response to an exacerbation of oxidative stress (altered glutathione homeostasis, accumulation of ROS, protein carbonylations, malondialdehyde and peroxidation/inflammatory products in the supernatants) [Gevi et al., 2012,

D'Alessandro et al., 2012]. Indeed, it is now widely accepted that long stored RBCs under blood bank conditions suffer from oxidative stress-triggered “storage lesions”, a series of either reversible or irreversible modifications to RBC morphology and biochemistry (both at the proteomic and metabolic level) [D'Alessandro et al., 2012, D'Amici et al., 2007]. Within this framework, in the transfusion milieu alternative storage strategies for RBC concentrates have been pursued with the goal to improve RBC quality and viability, and to extend the shelf life of erythrocyte concentrates up to 63 days, a goal that might be achieved through deoxygenation of RBC units [Dumont, et al., 2009, Yoshida and Shevkoplyas, 2010, Yoshida et al., 2007]. While these alternative storage strategies are currently under clinical testing [Yoshida and Shevkoplyas, 2010], it is yet to be assessed whether the observed alterations of the metabolic fluxes in RBCs upon deoxygenation are persistent throughout a 42 days storage period. Indeed only preliminary, albeit significant, information has been gathered by comparing a handful of metabolic parameters over a 42 days anaerobic storage period, including glucose consumption, glyceraldehyde 3-phosphate accumulation (via glycolysis or PPP) under high versus low oxygen saturated hemoglobin conditions, 14 ATP and 2,3-DPG [Scalbert et al., 2009], and lactate [Galtieri et al., 2002, Scalbert et al., 2009]. Taking advantage of recent technical improvements in the field of metabolomics [Scalbert et al., 2009], we hereby investigated the RBC metabolome of deoxygenated RBC units by means of a novel high performance liquid chromatography (HPLC)-micro-time of flight-quadrupole (micro-TOF-Q) mass spectrometry (MS) approach, a workflow that recently contributed precious insights into the understanding of RBC metabolism under control blood banking conditions [Gevi et al., 2012], or in pathological RBCs (e.g. hereditary stomatocytosis, sickle cell disease) [Darghouth et al., 2011, Darghouth et al., 2011, Darghouth et al., 2011].

3.2. DESIGN AND METHOD

3.2.1 Sample collection

Red blood cell units were drawn from healthy donor volunteers according to the policy of the Italian National Blood Centre guidelines (Blood Transfusion Service for donated blood) and upon informed consent in accordance with the declaration of Helsinki. We

studied RBC units collected from 10 healthy donor volunteers [male = 5, female = 5, age 39.4 ± 7.5 (mean $\pm$ S.D.)]. RBC units were stored for up to 42 days under standard conditions (CDP-SAGM, [D'Alessandro et al., 2011]), while samples were removed aseptically for the analysis on a weekly basis (at 0, 7, 14, 21, 28, 35 and 42 days of storage). Deoxygenation of erythrocyte concentrate units was achieved through 5 repeated gas exchange cycles consisting of bubbling high purity helium (Sol S.p.A. – Pomezia, Italy) under gentle agitation every 10 minutes at room temperature for 50 minutes, while maintaining sterile conditions, as previously reported [D'Amici et al., 2007]. Control counterparts were maintained under the same room temperature conditions in order to exclude any other bias to RBC metabolism than deoxygenation itself. Deoxygenated units were stored under a helium gas atmosphere under standard conditions (4 °C). Deoxygenation was confirmed through spectrophotometric assays in the range between 500 and 650 nm.

3.2.2 Materials

Acetonitrile, formic acid, and HPLC-grade water were purchased from Sigma Aldrich (Milan, Italy). Standards (equal or greater than 98% chemical purity) ATP, L -lactic acid, phosphogluconic acid, NADH, D-fructose 1,6-biphosphate, D-fructose 6-phosphate, glyceraldehyde phosphate, phosphoenolpyruvic acid, L-malic acid, L-glutamic acid, oxidized glutathione, and α -ketoglutarate were purchased from Sigma Aldrich (Milan). Standards were stored either at -25°C, 4°C or room temperature, following manufacturer's instructions. Each standard compound was weighted and dissolved in nanopure water. Starting at a concentration of 1 mg mL⁻¹ of the original standard solution, a dilution series of steps (in 18 MΩ, 5% formic acid) was performed for each standard in order to reach the determined linearity range for relative quantitation using peak areas.

3.3 UNTARGETED METABOLOMICS ANALYSES

3.3.1 Metabolite extraction

For each sample, 0.5 mL from the pooled erythrocyte stock was transferred into a microcentrifuge tube (Eppendorfs, Germany). Erythrocyte samples were then centrifuged at 1000g for 2 minutes at 4°C. Tubes were then placed on ice while

supernatants were carefully aspirated, paying attention not to remove any erythrocyte at the interface. Samples were extracted following the protocol by D'Alessandro et al.⁴² The erythrocytes were resuspended in 0.15 mL of ice cold ultra-pure water (18 M Ω) to lyse cells, then the tubes were plunged into a water bath at 37°C for 0.5 min. Samples were mixed with 0.6 mL of -20°C methanol and then with 0.45 mL chloroform. Subsequently, 0.15 mL of ice cold ultra-pure water were added to each tube and they were transferred to a -20 °C freezer for 2–8 h. An equivalent volume of acetonitrile was added to the tube and transferred to a refrigerator (4°C) for 20 min. Samples with precipitated proteins were thus centrifuged at 10 000 x g for 10 min at 4°C. Finally, samples were dried in a rotational vacuum concentrator (RVC 2–18 – Christ GmbH; Osterode am Harz, Germany) and re-suspended in 200 μ l of water, 5% formic acid and transferred to glass auto-sampler vials for LC/MS analysis.

3.3.2 Rapid resolution reversed-phase HPLC

An Ultimate 3000 Rapid Resolution HPLC system (LC Packings, DIONEX, Sunnyvale, USA) was used to perform metabolite separation. The system featured a binary pump and a vacuum degasser, a well-plate autosampler with a six-port micro-switching valve, a thermostated column compartment. Samples were loaded onto a Reprosil C18 column (2.0 mm x 150 mm, 2.5 μ m – Dr Maisch, Germany) for metabolite separation. Chromatographic separations were achieved at a column temperature of 30°C; and a flow rate of 0.2 mL min⁻¹. For downstream negative ion mode (-) MS analyses, a 0–100% linear gradient of solvent A (10 mM tributylamine aqueous solution adjusted with 15 mM acetic acid, pH 4.95) to B (methanol mixed with 10 mM TBA and with 15 mM acetic acid, pH 4.95) was employed over 30 min, returning to 100% A in 2 minutes and a 6 min post-time solvent A hold. For downstream positive ion mode (+) MS analyses, a 0–100% linear gradient of solvent A (ddH₂O, 0.1% formic acid) to B (acetonitrile, 0.1% formic acid) was employed over 30 min, returning to 100% A in 2 minutes and a 6 min post-time solvent A hold.

3.3.3 Mass spectrometry: Q-TOF settings

Due to the use of linear ion counting for direct comparisons against naturally expected isotopic ratios, time-of-flight instruments are most often the best choice for molecular

formula determination. Thus, mass spectrometry analysis was carried out on an electrospray hybrid quadrupole time-of flight mass spectrometer MicroTOF-Q (Bruker-Daltonik, Bremen, Germany) equipped with an ESI-Ion source. Mass spectra for metabolite extracted samples were acquired both in positive and in negative ion mode. ESI capillary voltage was set at 4500 V (+) (-) ion mode. The liquid nebulizer was set to 27 psi and the nitrogen drying gas was set to a flow rate of 6 L min⁻¹. The dry gas temperature was maintained at 200 °C. Data were stored in centroid mode. Data were acquired with a stored mass range of m/z 50–1200. Automatic isolation and fragmentation (AutoMSn mode) was performed on the 4 most intense ions simultaneously throughout the whole scanning period (30 min per run). Calibration of the mass analyzer is essential in order to maintain a high level of mass accuracy. Instrument calibration was performed externally every day with a sodium formate solution consisting of 10 mM sodium hydroxide in 50% isopropanol : water, 0.1% formic acid. Automated internal mass scale calibration was performed through direct automated injection of the calibration solution at the beginning and at the end of each run by a 6-port divert-valve.

3.3.4 Data elaboration and statistical analysis

In order to reduce the number of possible hits in molecular formula generation, we exploited the SmartFormula3D™ software (Bruker Daltonics, Bremen, Germany), which directly calculates molecular formulae based upon the MS spectrum (isotopic patterns) and transition fingerprints (fragmentation patterns). This software generates a confidence-based list of chemical formulae on the basis of the precursor ions and all fragment ions, and the significance of their deviations to the predicted intact mass and fragmentation pattern (within a predefined window range of 5 ppm). Triplicate runs for each one of the 10 biological replicates over storage duration were exported as mzXML files and processed through XCMS data analysis software (Scripps Centre for Metabolomics)⁴³ and MAVEN.⁴⁴ Mass spectrometry chromatograms were elaborated for peak alignment, matching and comparison of parent and fragment ions, and tentative metabolite identification (within a 20 ppm mass-deviation range between observed and expected results against the internal database – METLIN⁴⁵). XCMS and MAVEN are open-source software that could be freely downloaded from their websites

(<http://metlin.scripps.edu/download/> and <http://genomics.pubs.princeton.edu/mzroll/index.php?show=download>). Metabolite assignment was further elaborated in the light of the hydrophobicity/hydrophilicity of the compound and its relative retention time in the RP-HPLC run. Relative quantitative variations of intact mass peak areas for each metabolite assigned through MS/MS were determined against day 0 controls and only statistically significant results were considered (ANOVA p-values ≤ 0.01). Data were further refined and plotted with GraphPad Prism 5.0 (GraphPad Software Inc.).

3.4 RESULTS AND DISCUSSION

HPLC-MS runs were performed in triplicate on samples extracted from each donated unit at 0, 7, 14, 21, 28, 35, 42 days of storage.

In order to report the main results in a more readable layout, metabolites accounting for the most relevant catabolic pathways in RBCs were grouped and plotted as follows: metabolites involved in (i) glycolysis (Fig. 1), (ii) adenosine energy metabolism (Fig. 2), (iii) pentose phosphate pathway (PPP) and glutathione homeostasis (Fig. 3), (iv) purine salvage pathway (PSP – Fig. 4), (v) aminoacid transport and fatty acid/lipid metabolism (Fig. 5).

3.4.1 Glycolysis and energy metabolism are sustained throughout anaerobic storage duration

Under control storage conditions, prolonged glycolysis and lactate accumulation result in pH drop and, in turn, this triggers a negative feedback on the glycolysis rate itself. Indeed, glycolytic enzymes (for example the rate limiting enzyme phosphofructokinase) are inhibited by (i) low pH [Burger et al., 2010]; (ii) high concentrations of high energy phosphate compounds, such as ATP and DPG; (iii) selective binding to the CDB3 [Weber et al., 2004]. All these events are known to occur within the framework of RBC storage [D'Alessandro et al., 2010]. The rapid fall of glycolysis over storage duration has recently been detailed by Nishino and colleagues for PAGGM-stored RBCs and our group (SAGM-stored RBCs) [Gevi et al., 2012, D'Alessandro et al., 2012]. In deoxygenated RBC units, it is to be expected that these phenomena are mitigated to some extent, owing to the following reasons: (i) Hb is forced in the T-state (deoxy-Hb);

(ii) free H^+ ions are bound to deoxy-Hb as a result of the Bohr effect [Jensen, 2004]; (iii) high energy phosphates (mainly 2,3- DPG) are sequestered by deoxy-Hb at a near 1:1 stoichiometric ratio [Jensen, 2004]; (iv) competitive binding of deoxy-Hbs to the CDB3 dislocates and disinhibits glycolytic enzymes [Low et al., 1986, Gevi et al., 2012, Lewis et al., 2009, Castagnola et al., 2010, Messina, et al., 1996, Galtieri, et al., 2002]. Consistent with these assumptions, in anaerobically-stored RBCs we could observe a rapid increase in glucose consumption, that was paralleled by the accumulation of all glycolytic intermediates, including: glucose/fructose 6-phosphate (these isobaric species cannot be distinguished with MS, as previously reported [Gevi et al., 2012, Darghouth et al., 2011]), fructose 1,6-diphosphate, dihydroxyacetone phosphate, glyceraldehyde 3-phosphate, phosphoglycerate (though increasing significantly upon 35 days of anaerobic storage), phosphoenolpyruvate and lactate (Fig. 1).

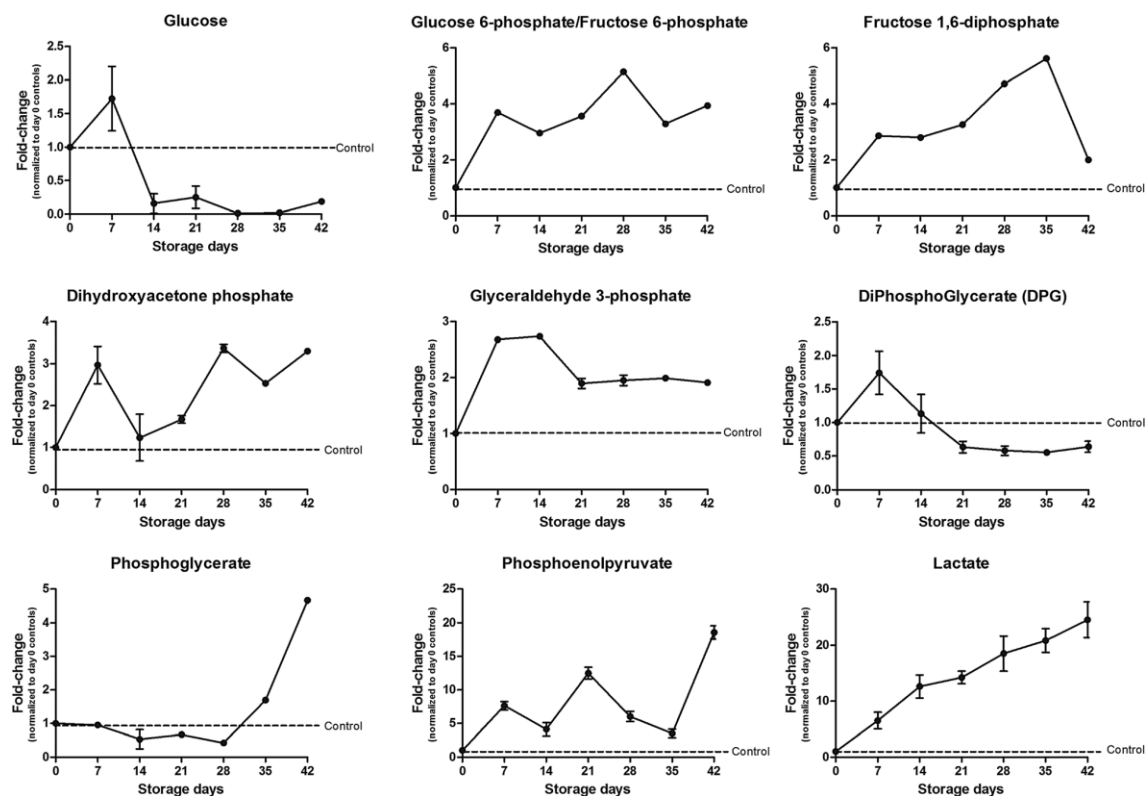


Figure 1—Time course metabolomic analyses of RBCs stored under anaerobiosis in SAGM additive solution at 4 1C up to 42 days. Results are plotted on a weekly basis (storage day 0, 7, 14, 21, 28, 35, 42) as in ref. 31, as fold-change variations upon normalization against day 0 controls. An overview of the trends for glycolytic metabolites. Averages and standard deviations were calculated on 10 biological replicates, each one assayed in triplicate runs at each storage day.

At the end of the anaerobic storage lactate increased by 24.55 ± 3.17 -fold in comparison to day 0 controls, which is consistent with previous reports by Yoshida's group [Yoshida and Shevkoplyas, 2010, Yoshida et al., 2007] and it is higher in comparison to our previous metabolomics investigation of RBCs stored under control (aerobic conditions) at the end of the storage period (42 days lactate being 20.48 ± 0.31 [Gevi et al., 2012]). Our results are in agreement with these observations (Fig. 1).

The glycolysis rate drops under control (aerobic) storage conditions, which results in ATP concentrations reaching a climax at 10–20% above the starting level within the first two to three weeks of storage, while they rapidly decline soon afterwards [Gevi et al., 2012, Yoshida and Shevkoplyas, 2010, Yoshida et al., 2007]. This is biologically relevant if we consider that the fall of ATP levels and of the total adenylate content (ATP + ADP + AMP) is associated with poor *in vivo* survival [Hogman et al., 1985], since the energy-less RBC is rapidly removed from the bloodstream. While these considerations hold true for aerobic RBC storage, under anaerobic conditions, Yoshida and colleagues reported that ATP peaks at higher levels (50–70% above the initial concentration), and this phase of ATP boost is sustained for a longer period (5–7 weeks) (though their observations also depended on the additive solutions used as a medium for RBC storage) [Dumont et al., 2009, Yoshida and Shevkoplyas, 2010, Yoshida et al., 2007]. Our results (Fig. 2) are consistent with these observations. It should also be noted that erythrocytes tend to release ATP as a vasodilator molecule in response to hypoxia [Dietrich et al., 2000], and ADP negatively regulates this process by acting on P2Y₁₃ receptors of human RBCs [Wang et al., 2005]. In the present study, we could observe that ADP levels decreased over storage duration, while AMP levels suffered from oscillations as they underwent both an early and late increase within the first and the last two weeks of storage (Fig. 2). On the other hand, cAMP levels increased throughout anaerobic storage duration, reaching an intermediate peak by day 21 and apexing by day 42 of anaerobic storage (Fig. 2). The increase in cAMP over storage duration is consistent with an ATP decrease despite sustained glycolysis in the long term, since cAMP accumulation under hypoxic conditions might trigger ATP release through signalling cascades involving cAMP-dependent protein kinase (PKA) and downstream activated pathways, such as the ones leading to mechanical deformation mediated by the activity of the cystic fibrosis transmembrane conductance regulator (CFTR) [Sprague et

al., 2001]. No significant shift towards the pentose phosphate pathway (PPP) despite moderate oxidative stress PPP accounts only for approximately 8% of glucose metabolism in RBCs under normal steady-state conditions, since 92% of glucose is metabolized through glycolysis (Embden–Meyerhof).

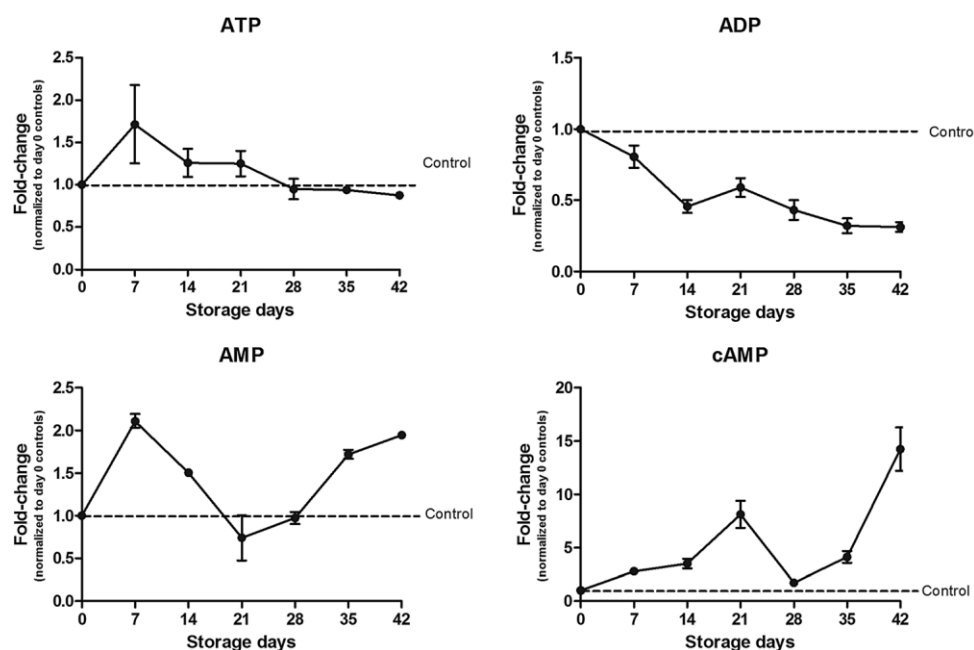


Figure 2—An overview of the trends for adenosine energy metabolism and related metabolites in time course analyses of RBC anaerobic storage on a weekly basis. Results are plotted as fold-change variations upon normalization to day 0 controls.

When faced with oxidative stress, RBCs respond to diverting as much as 90% of glucose metabolism toward the PPP [Messana et al., 1999, Misiti et al., 2002]. The rationale behind this phenomenon is in part explained by Rogers and colleagues, who envisage a role for the oxygen dependent modulation of glycolytic enzyme activity via competitive binding to the CDB3 and their availability to compete for glucose substrates with the PPP enzymes [Rogers et al., 2009]. Approximately 60% of the total NADPH production in humans relies on the PPP, as obtained through reduction of NADP^+ . This reaction is coupled to the formation of 6-phosphogluconolactone from glucose 6-phosphate and ribulose 5-phosphate from 6-phosphogluconate. The utter end-product of the oxidative phase is represented by ribulose 5-phosphate, that can further react with non-oxidative phase intermediates (carbon chain molecules ranging from 3 to 7 carbon atoms) and re-enter glycolysis at the glyceraldehyde 3-phosphate level, and/or

rather be recycled as fructose 6-phosphate, as an early glycolytic precursor. Since NADPH is needed to reduce the disulfide form of glutathione (GSSG) to the sulfhydryl form (GSH), an oxidative stress modulated increase in the PPP rate is to be considered a natural self-defensive mechanism of erythrocytes to cope with oxidative injury, as also deducible from widespread hereditary anomalies to rate limiting enzymes of this pathways [Efferth et al., 2004, Fico et al., 2004]. In 1999, Dumaswala et al. demonstrated that GSH and glutathione peroxidase provide the primary antioxidant defense in stored RBCs, and their decline, concurrent with an increase in oxidative modifications of membrane lipids and proteins, is tied to the destabilization of the membrane skeleton and thus to a compromised RBC survival [Dumaswala et al., 1999]. Indeed, GSH is pertinent for maintaining the normal structure of RBCs and for keeping haemoglobin in the ferrous state [Fe(II)]. Recently, we observed that storage of RBC under control aerobic conditions resulted in a metabolic diversion towards the oxidative phase of the PPP in the short term (after the second week of storage), while metabolic end-products of the nonoxidative phase of the PPP appeared to serve as substrates for PSP reactions, rather than for massively re-entering glycolysis [Gevi et al., 2012]. This observation was consistent with an early increase in glyceraldehyde 3-phosphate production via the PPP early upon exposure to high oxygen saturation conditions, while differences with erythrocytes exposed to low oxygen saturation conditions were attenuated in the long term [Messana et al., 1999]. In the present study, we observed no evident increase in PPP oxidative phase intermediates, while isobaric species in the non-oxidative phase of the PPP suffered from an early decline below control levels yet by anaerobic storage day 7 (ribulose 5-phosphate, xylose 5-phosphate, ribose 5-phosphate, ribose 1-phosphate – could not be distinguished through the current MS approaches, in agreement with Kinoshita et al., 2007 and Gevi et al., 2012) (Fig. 3). The blockade of the storage-dependent metabolic diversion towards the PPP is in line with the observations by Rogers and colleagues about short term RBC exposure to hypoxia [Rogers et al., 2009]. GSH is synthesized through a two step ATP-dependent process: in the first and rate limiting step, the enzyme gamma-glutamylcysteine synthetase exploits the aminoacid substrates, glutamate and cysteine to generate gamma-glutamylcysteine; in the second step, glycine is added by glutathione synthetase [Lu 2009]. The levels of

glutamine (precursor to glutamate – oscillating trend) increased over anaerobic storage (Fig. 3).

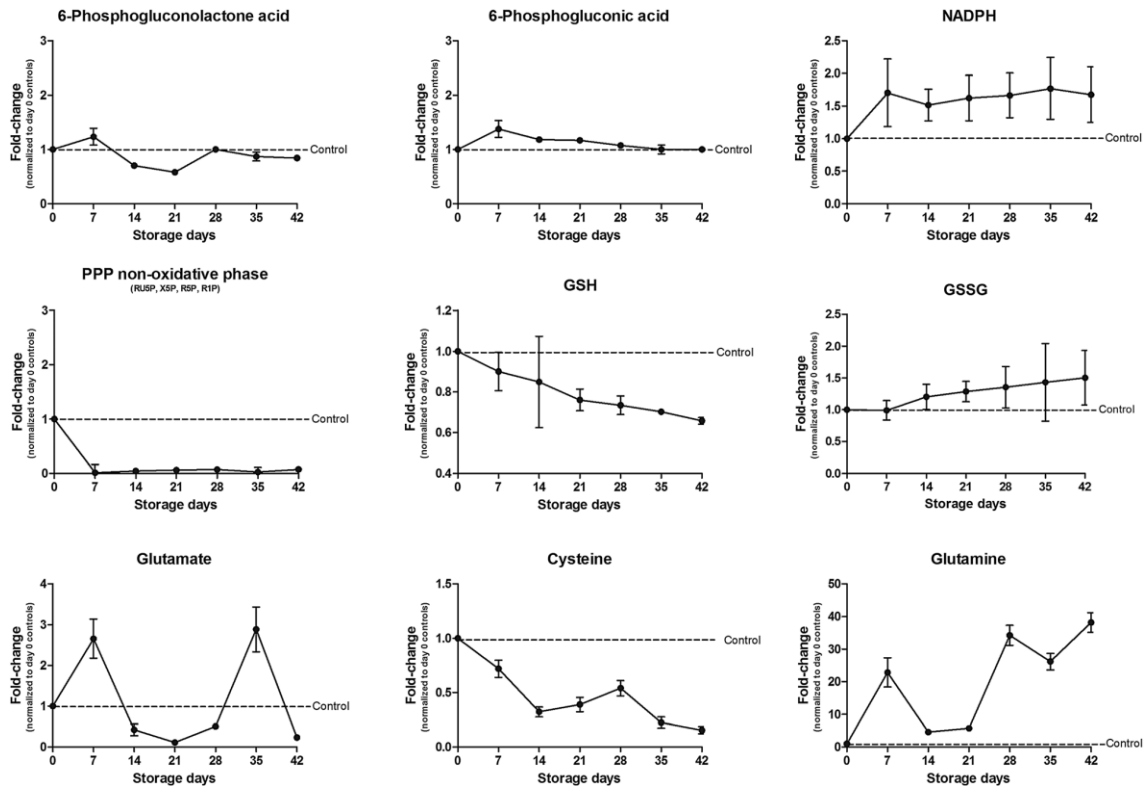


Figure 3-An overview of the trends for the pentose phosphate pathway and glutathione homeostasis during time course analyses of RBC anaerobic storage on a weekly basis. Results are plotted as fold-change variations upon normalization to day 0 controls.

Conversely, free cysteine decreased over anaerobic storage duration (Fig. 3). It is interesting to note that HEK293 and Hep3B cells exposed to 1.5% O₂ exhibit a time-dependent decrease in cellular glutathione stores and concomitant inhibition of glutathione biosynthesis, which correlates to impaired transport of the substrate cysteine [Mansfield et al., 2004]. Also, anoxia impairs cysteine-dependent GSH biosynthesis more than hypoxia in hepatocytes owing to lower ATP production via mitochondria [Shan et al., 1989]. However, it is worthwhile to stress that, unlike hepatocytes, RBCs do no longer have mitochondria. Therefore, oxygen removal appeared not to completely eliminate oxidative stress, as suggested by the following observations: (i) the moderate albeit constant decrease in GSH and (ii) increase in GSSG levels (Fig. 3); (iii) the accumulation of inflammation/oxidative stress-related markers such as prostaglandin

D2/E2 and thromboxane A₂ [Ibe et al., 1987]; and (iv) the decrease in antioxidant exogenous compounds such as catechins and epicatechins⁶³ (data not shown). On the other hand, the oxidative stress-marker prostaglandin F_{2a} (8-isoprostane⁶⁴) decreased significantly over anaerobic storage duration (data not shown). First of all, it is to be excluded that the observed phenomena are to be attributed to partial deoxygenation (hypoxia instead of anoxia), since deoxy-Hb spectrophotometric spectra were assayed and confirmed in the 500–650 nm range. Indeed, enhanced rates for the formation of ROS and RNS occur under hypoxic conditions where an increased fraction of Hb is bound to the RBC membrane [Rifkind and Nagababu, 2012]. Under normoxic RBC storage conditions, Hb migration to the membrane fraction is accompanied by increased membrane-bound levels of active peroxiredoxin 2 [Rinalducci et al., 2011], which binds to the same membrane docking site (i.e. the CDB3 [Matte et al., 2012]) thereby mitigating membrane targeting oxidative stress. However, peroxiredoxin 2 migration to the membrane does not occur during storage under anaerobic conditions [Rinalducci et al., 2011], which might imply that oxidative stress is not as much sustained as in normoxic counter-parts as to activate certain anti-oxidant defensivemechanisms. It should also be noted that, under hypoxic (not anaerobic) conditions, when Hb is partially saturated with oxygen, the oxygen is constantly leaving one Hb molecule and binding to another. Thus, intermediate oxygen pressures and hypoxic conditions in RBCs favour the production of superoxide radicals from Hb-bound oxygen, owing to enhanced heme pocket flexibility and higher interactions with distal histidine, which in turn promotes the destabilization of the iron–oxygen bond and in the release of superoxide radicals [Balagopalakrishna et al., 1996]. In this view, anaerobic storage strategies failing to achieve complete Hb deoxygenation should take this phenomenon into serious account. A rationale behind the incomplete elimination of oxidative stress under anaerobic conditions might stem from considerations about the interplay of pro-oxidant/anti-oxidant mechanisms in RBCs [Tsikas et al., 2012]. Tsikas and colleagues recently reported that GSH promoted the concomitant formation of the current oxidative stress biomarkers malondialdehyde (MDA) and prostaglandins from arachidonic acid via prostaglandin H synthases. On this ground, it emerges that uncommon interplay of enzymatic and chemical reactions might yield species that are considered to be exclusively produced by free-radical-catalysed reactions [Tsikas et al., 2012].

3.4.2 Purine salvage pathway is activated proportionally to storage progression even under anaerobic conditions

Since mature RBCs are incapable of de novo synthesizing 5-phosphoribosylamine, they rely on salvage pathways to restore purine levels [Schuster and Kenanov, 2005]. Adenine and adenosine are transported through the erythrocyte membranes through the facilitated diffusion [Hess, 2010]. They are the precursors of adenine nucleotides, accounting for 70–80% of all free erythrocyte nucleotides [Schuster and Kenanov, 2005, Hess, 2010]. Anaerobic storage resulted in an increase in intracellular adenine levels, while adenosine followed oscillating trends (despite being comparable to day 0 controls by 42 days of storage – Fig. 4). Hypoxanthine and inosine are two major substrates for salvage reactions [Schuster and Kenanov, 2005, Zolla et al., 1972]. While hypoxanthine levels oscillated throughout anaerobic storage, inosine levels increased significantly, proportionally to anaerobic storage progression. Altered inosine levels in particular might result from deamination of adenosine, which is known to occur under control RBC storage conditions [Hess 2010]. Inosine formed in the adenosine deaminase reaction or supplemented to RBC via storage solutions may enter the erythrocyte and undergo phosphorolysis to form hypoxanthine and ribose 1-phosphate (R1P) [Gabrio et al., 1956]. Through this reaction it is possible to introduce a phosphorylated sugar (PPP non-oxidative phase intermediate) into the erythrocyte without ATP consumption [Gabrio et al., 1956]. The use of inosine has received much attention in the field of blood banking since it appears to be the only practical means to obtain ATP production in the cell without first expending ATP to prepare an unphosphorylated substrate for further metabolism [Gabrio et al., 1956]. Adenosine–homocysteine, which serves as a substrate to produce adenosine and homocysteine, accumulated in long anaerobically stored RBCs, while homocysteine decreased (Fig. 4). Homocysteine is also a precursor to cysteine (also decreasing – Fig. 3) and thus has an indirect role in GSH synthesis [Filip et al., 2012]. Anomalies to homocysteine fine-tuning are known to be related to oxidative stress [Filip et al., 2012] and deficiency B-group vitamins [Curtis et al., 1994]. However, no substantial alteration of vitamin intake was observed (retinol – vitamin A, riboflavin – vitamin B2 – Data not shown).

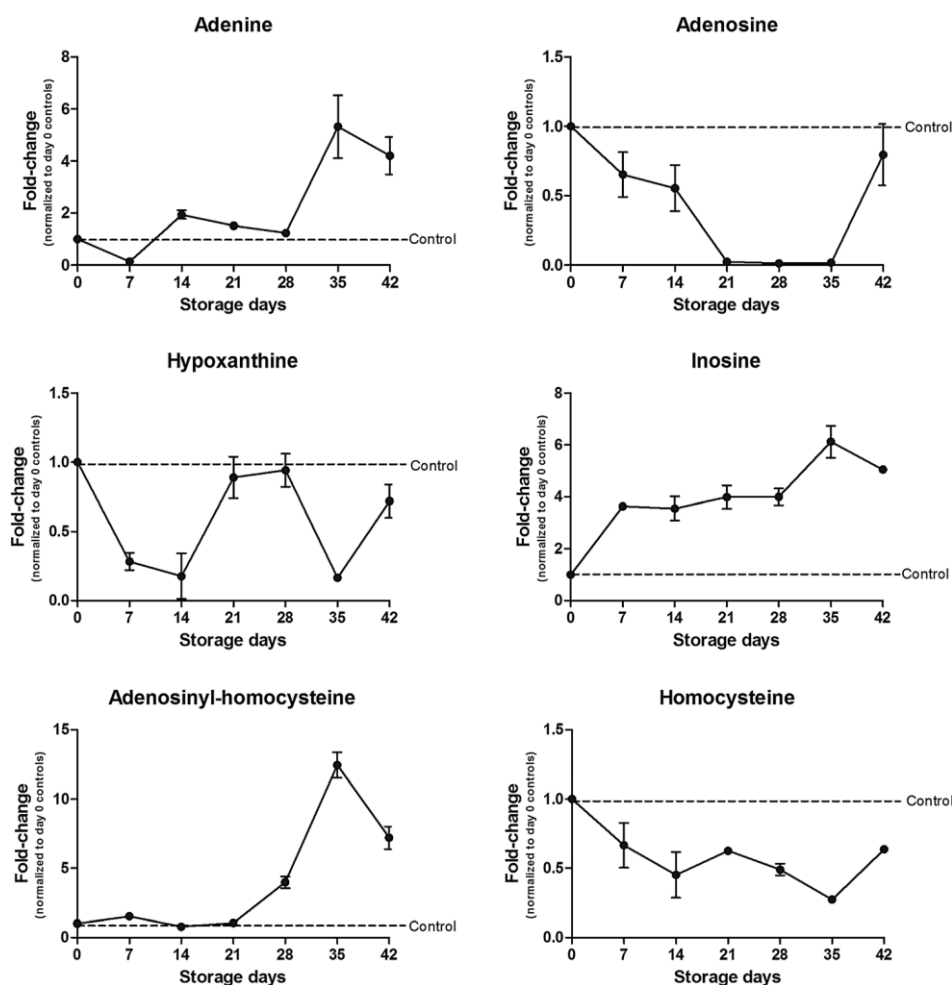


Figure 4 Purine salvage pathway main metabolites during RBC anaerobic storage on a weekly basis. Results are plotted as fold-change variations upon normalization to day 0 controls.

3.4.3 Amino acid transport and lipid homeostasis are affected to some extent under anaerobic storage

Though RBC metabolism has been hitherto supposed to be restricted to pathways enlisted in the previous paragraphs (glycolysis, PPP, PSP, Rapoport–Luebering, methemoglobin reduction pathway), recent advancements in the understanding of the RBC proteome suggested that erythrocytes might also rely on yet undisclosed/uncharacterized pathways [D'Alessandro et al., 2010, Roux-Dalvai Gonzalez et al., 2008]. In a recent report, Darghouth and colleagues indicated that 30.4% of the RBC metabolome (including 89 validated metabolites) was made up of free aminoacids. The capillary distribution of RBCs through the cardiovascular system allows RBCs to intake aminoacids from plasma, store and deliver them to those districts where they are needed

the most. In this view, RBCs might represent active vessels in the inter-organ transport [Elwyn et al., 1972].

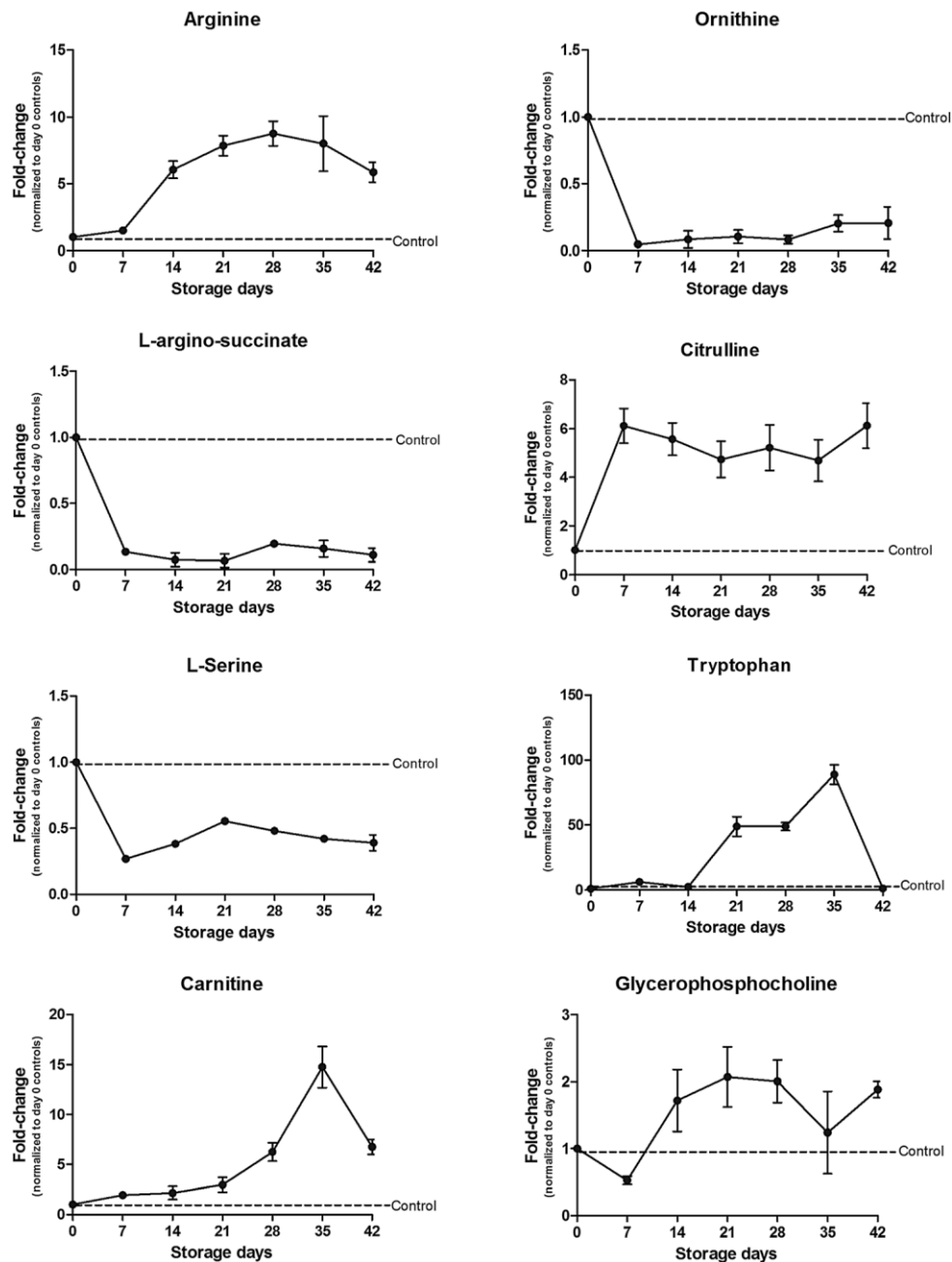


Figure 5—A glance at amino acid transport and fatty acid metabolism in anaerobically stored RBCs. Results are plotted as fold-change variations upon normalization to day 0 controls.

Owing to the lack of nuclei and ribosomes, RBCs are devoid of any protein synthesis capacity. Nonetheless, numerous aminoacid transport systems have been found in human RBCs in analogy to other cell types, and anomalies to these RBC aminoacid

transport systems have been related to several diseases, including chronic renal failure [Divino Filho et al., 1997, Canepa et al., 2002]. Free arginine levels increased over anaerobic storage, while ornithine decreased (Fig. 5). While arginase-mediated conversion of arginine to ornithine and urea mainly occurs in liver, arginase is also present in RBCs [Kim et al., 2002]. In circulating RBCs, arginine could be imported from plasma to RBCs through the y^+ system [Tunncliffe, 1994], or might be produced in RBCs from aspartate as demonstrated in analogy to other cell types [Kanehisa and Goto, 2000]. Arginine might be produced from citrulline and argino-succinate, while the metabolic pathway might shift back to citrulline via nitric oxide synthase (which is present and functionally active in RBCs [Kleinbongard et al., 2006]), resulting in the production of NO. This is particularly relevant in the frame of deoxygenated districts, where deoxy-Hb binds to NO• thus functioning as a transporter and/or reduces nitrite to generate NO• promoting vasodilation [Filip et al., 2012]. Consistently, while argino-succinate decreased in a similar fashion to ornithine, anaerobic storage promoted the accumulation of both arginine and citrulline (Fig. 5). These results might contribute to justify the observation about oxidative stress in long stored deoxygenated RBC units. Phosphoglycerate dehydrogenase, a key enzyme in serine metabolism [Locasale et al., 2011], has been reported in the most recently updated report about the RBC proteome [D'Alessandro et al., 2010]. L-Serine decreased yet upon 7 days of anaerobic storage, while its concentrations remained apparently unaltered thereon (Fig. 5). Tryptophan transport in RBCs has been investigated during the last three decades [Rosenberg et al., 1980], owing to its implications in the pathogenesis of depressive disorders [Jeanningros et al., 1996]. Tryptophan uptake increased in the time window range between 21 and 35 days of storage, though at the end of the storage intracellular tryptophan levels were comparable to those in day controls (Fig. 5). While mature RBCs are incapable of synthesizing lipids anew [Percy et al., 1973], alterations to lipid homeostasis are strictly tied to membrane blebbing leading to vesiculation and eryptosis [Lang et al., 2012], a phenomenon that is known to be exacerbated by ceramides. Ceramide may be produced from cell membrane sphingomyelin by a sphingomyelinase [Dinkla et al., 2012]. At the end of the anaerobic storage we could find that, in comparison to day 0 controls, day 42 RBCs displayed higher levels of sphingomyelins and ceramides, other than sphingosines (SM(d18:1/0:0); CerP(d18:1/ 24:0); C-2

ceramide; C-8 ceramide-1-phosphate; C-8; CerP(d18:1/ 12:0); ceramine; D-erythro-sphingosine C-15; glucosylsphingosine; phytosphingosine; sphinganine – Data not shown, analogously to day 42 (aerobically stored) control RBCs [Gevi et al., 2012]. Carnitine plays a buffer function in mediating the role of acyl-L-carnitine as a reservoir of activated acyl groups in mature human erythrocytes [Arduini et al., 1992]. This might relate to the increased concentrations of cholines in longer (anaerobically) stored RBCs (such as glycerophosphocholine – PC(O-18:1(10E)/2:0); PC(O-18:1(11Z)/0:0); PC(P-18:0/0:0); PC(O-16:0/6:0); PC(10:0/4:0) –), analogously to RBCs stored under normoxic blood banking conditions [Gevi et al., 2012]. Increased concentrations of cholines (especially in the intermediate storage period within 14 and 28 days of anaerobic storage – Fig. 5) have already been reported in sickle RBCs, where they have been linked to recycling of phospholipids as to indirectly document the RBC membrane fragility observed in sickle cell disease patients [Darghouth et al., 2011].

3.5 CONCLUSION

MS-based metabolomics is a promising research endeavor that is gradually complementing decades of accurate biochemical observations with targeted approaches and NMR [Nicholson and Lindon, 2008, D'Alessandro and Zolla, 2012, Wishart et al., 2009]. In the present study, the application of novel MS-based metabolomics approaches to the issue of RBC exposure to prolonged anaerobiosis has further shed light on the oxygen-dependent metabolic modulation in RBCs. Long term exposure of RBCs to anaerobic conditions resulted in the exacerbation of all those phenomena observed in the short term [Kinoshita et al., 2007]. We hereby confirmed previous evidence about long term anaerobiosis promoting glycolytic metabolism in RBCs and prolonging the conservation of high energy phosphate reservoirs and purine homeostasis [Dumont et al., 2009, Yoshida and Shevkoplyas, 2010, Yoshida et al., 2007]. ATP decrease despite glycolysis being sustained throughout the whole anaerobic storage duration (glycolytic intermediates and lactate accumulate constantly until storage day 42) might not only reflect ATP and DPG sequestering by deoxy-Hb, which should occur early upon deoxygenation, but it might rather reflect cAMP-mediated ATP release by RBCs in response to deoxygenation, a phenomenon that occurs in vivo to promote vasodilation in hypoxic districts [Dietrich et al., 2000]. In parallel, we

evidenced that, in contrast to aerobic storage [Gevi et al., 2012], anaerobiosis impairs RBC capacity to cope with oxidative stress by blocking metabolic diversion towards the PPP, which negatively affects glutathione homeostasis. Therefore, although oxidative stress is less sustained than in aerobically stored RBCs [Gevi et al., 2012], oxidative stress markers still accumulate over anaerobic storage progression, though to a lesser extent. In this view, it should be pointed out that, in the present study, anaerobiosis has been reached in SAGM-stored RBCs (pH_{22°C} 5.7), while higher pH additive solutions should in part counteract the PPP blockade by increasing glucose 6-phosphate dehydrogenase activity [Cohen and Rosemeyer, 1968]. Indeed, neutral or alkaline storage solutions have already been proposed with encouraging results (higher DPG and ATP levels at the end of the shelf-life, higher in vivo survival upon transfusion), both in the frame of aerobic [Hess et al., 2002, Veale 2011 et al., 2011] and anaerobic storage of RBCs [Dumont et al., 2009, Yoshida et al., 2008]. While metabolomics is one key area of biological investigations, further clues about the effects of prolonged exposure of RBCs to anaerobiosis will soon derive from the application of other omics disciplines, above all proteomics, to this delicate topic.

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CHAPTER 4:
THIOL-BASED REGULATION OF GLYCERALDEHYDE-3-PHOSPHATE
DEHYDROGENASE IN BLOOD BANK-STORED RED BLOOD CELLS:
A STRATEGY TO COUNTERACT OXIDATIVE STRESS

4.1 AIM OF THE STUDY

A growing body of evidence argues that regulation of red blood cell (RBC) energy metabolism indirectly depends on the assembly of some glycolytic enzymes (GEs; phosphofructokinase, aldolase, glyceraldehyde-3-phosphate dehydrogenase [GAPDH], lactate dehydrogenase [LDH]) into complexes associated with the cytoplasmic domain of the membrane protein Band 3 (CDB3) [Campanella et al., 2005, Lewis et al., 2009, Castagnola et al., 2010]. In particular, it has been demonstrated that when bound to CDB3, GEs are inhibited in their activity, while their release from the membrane to the cytoplasm results in their activities being restored and, thus, in metabolic modulation (Fig. 1) [Campanella et al., 2005, Lewis et al., 2009]. The Band 3-mediated metabolic regulatory model is also strictly intertwined with the hemoglobin (Hb) oxygenation state, because whereas oxy-Hb has no affinity for Band 3, deoxy-Hb binds to the GE site on CDB3 with high affinity [Chu et al., 2008]. Consequently, competition between deoxy-Hb and GEs for CDB3 causes GEs to be displaced from membrane with following activation and increased glycolysis (Fig. 1) [Campanella et al., 2005, Giardina et al., 1995, Messana et al., 1996, De Rosa et al., 2008]. Furthermore, tyrosine phosphorylation of CDB3 at Positions 8 and 21 competes with GE binding as well [Campanella et al., 2005], thus inducing an increased glycolytic flux and a reduced pentose shunt activity (Fig. 1) [Lewis et al., 2009]. Beyond any doubt, oxygen-dependent modulation of glucose metabolism in human RBCs has physiologic relevance because of its connection to the regulation of intracellular levels of NADPH and ATP. Over the past few years special attention has been devoted, in the framework of transfusion medicine, to the metabolic flux alterations in stored RBCs with the goal to improve cell survival and maintain good-quality RBC units up to the end of 42-day shelf life. Indeed, nowadays it is well known that storage lesions include metabolic effects such as potassium leakage, depletion of 2,3-diphosphoglycerate (2,3-DPG) and ATP, pH decrease, and lactate accumulation [D'Alessandro et al., 2010, Gevi et al., 2012]. In particular, under standard blood bank conditions (cold storage in the acidic SAGM), RBCs face a rapid decrease of the glycolytic rate with a shift toward the oxidative phase of the pentose phosphate pathway (PPP) [Gevi et al., 2012]. As observed *in vivo* [Murphy 1960], this strategy is adopted in response to an exacerbation of oxidative stress, due to altered glutathione homeostasis and reactive oxygen species

accumulation [D'Alessandro et al., 2012]. A central role in the stress-induced partial block of glycolysis may be played by the enzyme GAPDH that converts glyceraldehyde 3-phosphate (GAP) to 1,3-DPG. This reaction is considered a key regulatory point in the glycolysis because it generates the reducing power (NADH) to maintain Hb in its functional state. Unfortunately, GAPDH is extremely sensitive to oxidative radicals with subsequent loss of enzyme activity. Elementary flux mode analysis indicated that GAPDH deficiency causes cessation of all possible reaction routes within human RBCs [Cakir et al., 2004], thereby irreversibly compromising RBC viability. Accordingly, RBC deficiency of GAPDH has been described in association with hemolytic anemia [Harkness 1966]. However, temporary inactivation of GAPDH due to reversible oxidative modifications of the active-site thiolate (e.g., S-thiolation and intramolecular disulfide cross-linking) is known to function as a metabolic switch to redirect carbohydrate flux from glycolysis to PPP, allowing the cell to produce more NADPH in an attempt to maintain oxidation-reduction balance and protect itself against oxidative insults [Brandes et al., 2009, Ralser et al., 2007]. Based on these considerations, in this study we followed the redox regulation of RBC GAPDH during blood storage under control conditions (4°C, SAGM). Thus, membrane and cytosol fractions from stored RBCs were analyzed separately by Western blotting for the presence of GAPDH and assayed for catalytic activity. We revealed a time-dependent loss of membrane-bound GAPDH with a consequent increase of activity in the cytosol within the first two storage weeks, while a sharp decline in the cytosolic GAPDH activity was detected after 21-day storage, although we observed a restoration in enzyme activity toward the end of preservation. Moreover, by mass spectrometry (MS) analysis we were able to demonstrate the oxidant-mediated formation of an intramolecular disulfide bond between Cys-152 and Cys-156 as being the molecular mechanism responsible for the reversible inactivation of cytosolic RBC GAPDH.

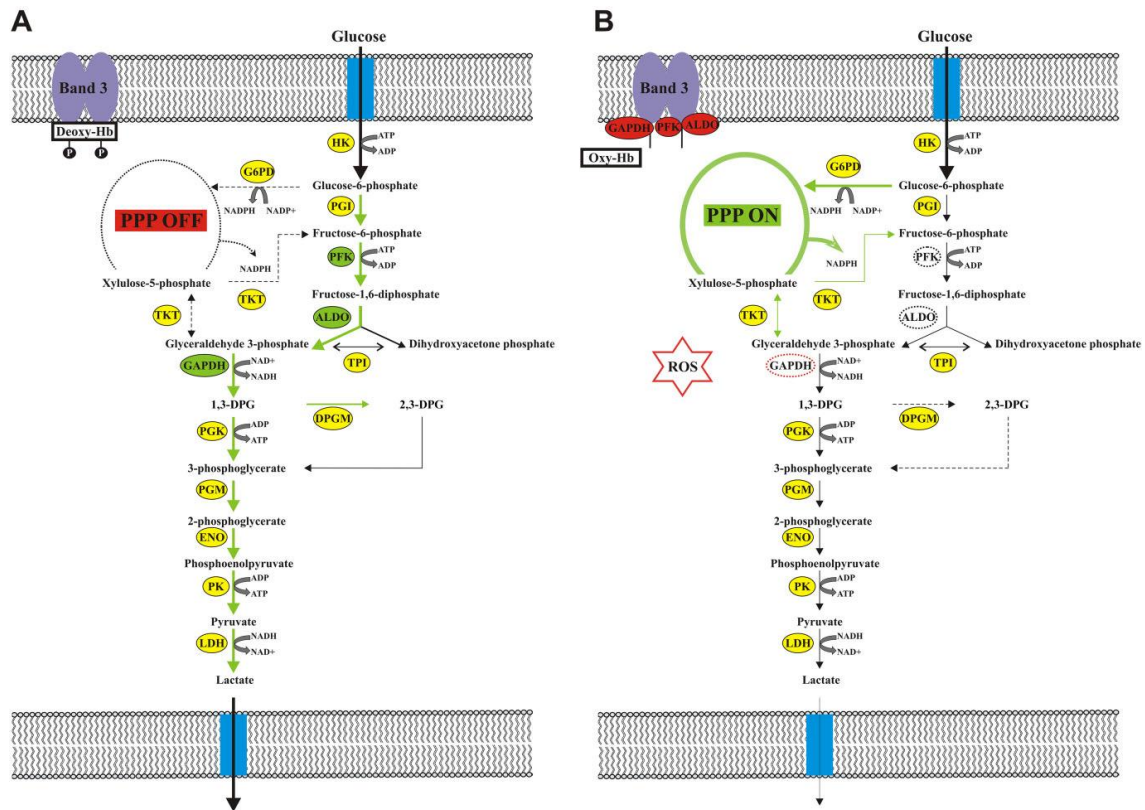


Figure 1-Schematic view of oxygen-dependent modulation of RBC metabolism. This regulatory model accounts for the competitive binding of deoxy-Hb and GEs phosphofructokinase, aldolase, and GAPDH to the CDB3. (A) When the RBC is at low oxygenation state, deoxy-Hb binds to the CDB3 and dislocates GEs, thus promoting their activation and subsequent increase of glucose flux toward the glycolytic pathway. This metabolic behavior may be consequence of CDB3 phosphorylation as well. (B) In response to high oxygen saturation conditions and oxidative stress, GEs are mostly bound to the CDB3 and thereby inhibited. This mechanism addresses more glucose toward the PPP with increased NADPH production. HK = hexokinase; PGI = phosphoglucose isomerase; PFK = phosphofructokinase; ALDO = aldolase; TPI = triose phosphate isomerase; PGK = phosphoglycerate kinase; DPGM = diphosphoglycerate mutase; PGM = phosphoglycerate mutase; ENO = enolase; PK = pyruvate kinase; G6PD = glucose-6-phosphate dehydrogenase; TKT = transketolase.

4.2 MATERIALS AND METHODS

4.2.1 Storage of RBCs and sample preparation

For this study RBC units were collected from five healthy donors (three males, two females; age, 35 ± 10.2 years [mean $\pm$ SD]) in Rome (Italy) and used according to the policy of the Italian National Blood Centre guidelines. All the volunteers provided their informed consent in accordance with the Declaration of Helsinki. Whole blood ($450 \text{ mL} \pm 10\%$) was collected into CPD (63 mL). After separation of plasma and buffy coat by centrifugation, leukofiltered RBCs were suspended in 100 mL of SAGM. RBC units were stored for up to 42 days under standard blood bank conditions ($4 \pm 2^\circ\text{C}$) and samples were collected aseptically for the analysis every week from Day 0 until Day 42

of storage. RBC protein extraction was performed exactly as previously described by Rinalducci et al [Rinalducci et al., 2011].

4.2.2 GAPDH assay

Enzymatic activity was determined using a colorimetric assay kit (Science Cell Research Laboratories, Carlsbad, CA) by measuring the rate of NADH oxidation, which is proportional to the reduction in absorbance at 340 nm over time ($\Delta A_{340\text{nm}}/\text{min}$). To control for possible loss of activity during sample handling and processing, spike and recovery experiments were performed by adding 0.2 units/mL standard pure GAPDH to the cell lysis buffer used for protein extraction. GAPDH activity was then measured in the cytosolic extract, in the standard solution (positive control) and in the mix. Spike recovery was 88% to 106%.

4.2.3 Western blotting analysis

After sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) separation (11% acrylamide for cytosolic samples and 8%-16% acrylamide for membrane samples), RBC proteins were transferred onto a nitrocellulose filter. Blocking was performed overnight (+4°C) in 1% (wt/vol) nonfat dried milk, 1% (wt/vol) bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20. Incubation with mouse polyclonal antibodies against human GAPDH (sc-137179, Santa Cruz Biotechnology, Dallas, TX) was performed for 1 hour at room temperature in 5% (wt/vol) nonfat dried milk in Tris-buffered saline and 0.1% Tween 20. Bands were detected with goat antimouse horseradish peroxidase using enhanced chemiluminescence reagents and digitized by an advanced imaging system (ChemiDoc XRS+ system, Bio-Rad, Hercules, CA).

4.2.4 Statistical analysis

Data were analyzed with computer software (GraphPad Prism 4.0 software, GraphPad, San Diego, CA) using oneway analysis of variance (ANOVA) followed by Dunnett's test. Results were considered significant when p values were less than 0.05.

4.2.5 In-gel digestion and peptide sequencing by nano-LC-ESI Q-TOF MS/MS

Protein bands observed in SDS-PAGE were carefully excised and subjected to in-gel trypsin digestion according to Shevchenko and colleagues [Shevchenko et al., 2006]. Nano-LC MS/MS analyses were performed as described in detail by Egidi and colleagues [Egidi et al., 2011]. Data were searched against the National Center for Biotechnology Information nonredundant database (NCBI nr, Version 20140323, 38032689 sequences) using computer software (Mascot, in-house version 2.2, Matrix Science, London, UK) with the following constraints: taxonomy = human; enzyme = trypsin; missed cleavage = 1; peptide and fragment mass tolerance = ± 0.05 Da; variable modifications = Deydro (Cys); Nethylmaleimide (Cys). For positive identification, the score of the result of $[-10 \times \text{Log}(P)]$ had to be over the significance threshold level ($p < 0.05$). Peptide fragmentation spectra were also manually verified.

4.3 RESULTS

4.3.1 Effects of RBC storage on GAPDH activity

As it is known, RBC GAPDH is inactive in its membrane-bound state, since this binding site (specific for CDB3) is located in the catalytic domain of the protein [Tsai et al., 1982]. Thus, the enzymatic activity of GAPDH was measured in the cytosol of RBCs at 0, 7, 14, 21, 28, 35, and 42 days of storage (Fig. 2A). To cope with biologic variability, the same analysis was performed on RBC units collected from five different matched donors and results are displayed as mean $\pm$ SD. The GAPDH activity is shown to increase within the first storage days, likely corresponding to an initial burst of glycolysis ensuing the internalization of extracellular glucose primarily found in the additive storage solution SAGM. Interestingly, this immediate increase in the GAPDH activity is followed by a net decrease at 21 storage days with a subsequent reestablishment of activity at the end of preservation time.

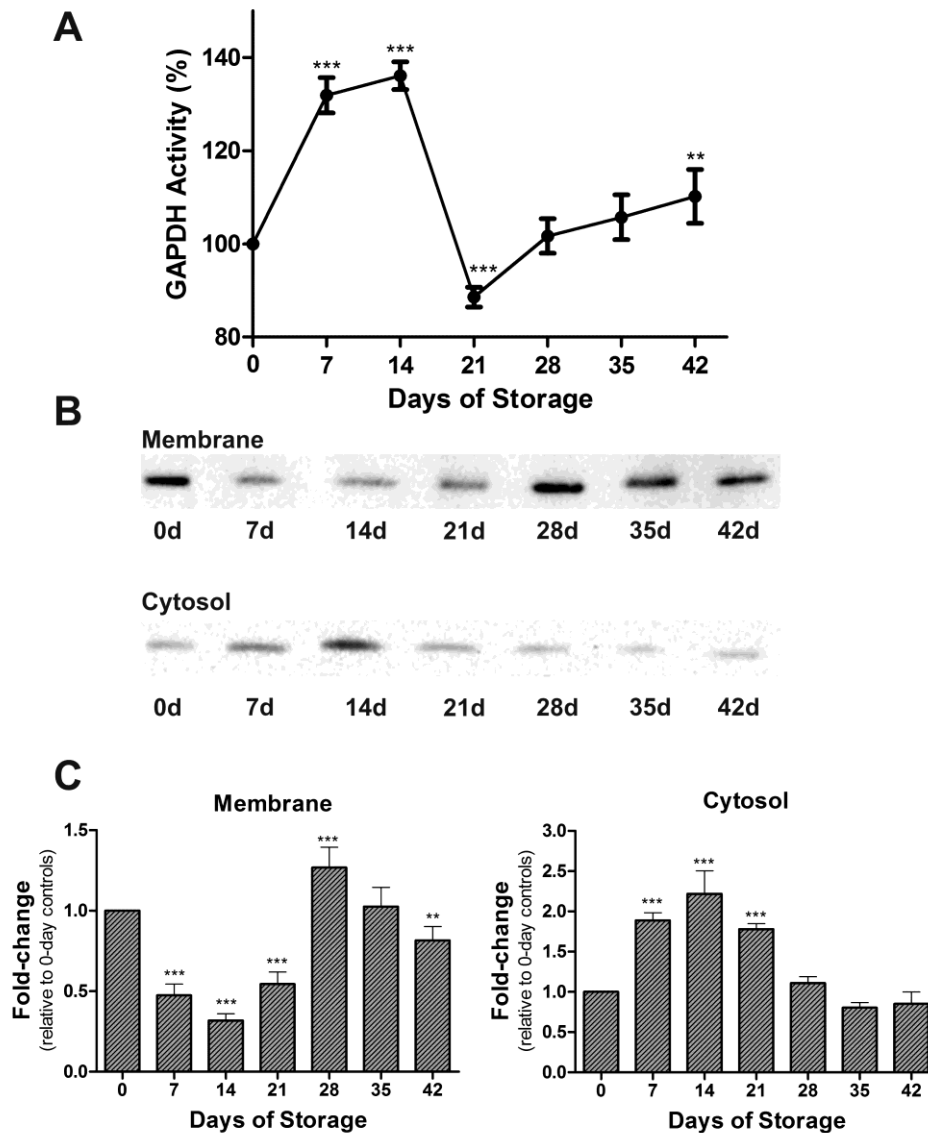


Figure 2-(A) Effects of storage time on cytosolic GAPDH activity of RBCs under standard blood bank conditions. The enzyme activity was expressed as a percentage of control (0 days of storage) and corrected for the Hb content, measured as absorbance at 412 nm. The activity measurements performed at Day 0 showed a coefficient of variation of 3%. Each data point represents the mean $\pm$ SD of the GAPDH activity for the tested RBC units ($n = 5$) estimated by independent measurements done in triplicate. Asterisks above the error bars denote significant differences versus control (one-way ANOVA with Dunnett's correction). ** $p < 0.01$; *** $p < 0.001$. (B) Immunoblotting of membrane-bound and cytosolic GAPDH from stored RBCs. Proteins were separated by SDS-PAGE under reducing conditions (solubilization buffer, 0.125 mol/L Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.053% bromophenol blue, 200 mmol/L dithiothreitol). The blot is representative of five similar experiments. (C) Densitometric analysis of GAPDH immunoblots. Individual protein levels were quantified after normalization to the total blotted proteins per day of storage and expressed as ratio versus control (Day 0). Data are presented as mean $\pm$ SD ($n = 5$, different donors). Statistics and symbols are as in A.

4.3.2 Storage-dependent displacement of RBC GAPDH from membrane to cytosol

Due to the well-known correlation between the GAPDH activity and the cellular localization of this protein, we investigated the storage-dependent release of the GE from RBC membranes to cytosol, in the attempt to explain the changes observed in the enzymatic activity described previously. GAPDH has been demonstrated to be largely located at the membrane level in unstimulated, healthy, or oxygenated RBCs [Campanella et al., 2005]; thus this conceivably represents our situation at 0-day storage (freshly drawn RBCs). To verify this assumption, we performed a semiquantitative Western blot analysis of RBC membranes and cytosol by using anti- GAPDH. Figure 2B and 2C shows the results obtained at 0, 7, 14, 21, 28, 35, and 42 days of storage. As can be seen, a time-dependent GAPDH release from the membrane was observed within the first 3 weeks of storage, in agreement with the initial storage-induced activation of the enzyme (as reported in Fig. 2A). These findings suggest that most of the membrane-bound GAPDH is activated early during storage through a displacement into the cytosol. Curiously, a new membrane binding of the protein is observed toward the end of storage time.

4.3.3 Identification of posttranslational oxidative modifications responsible for GAPDH inactivation

GAPDH is extremely sensitive to the modification of the cysteine residue (Cys-152) located in its catalytic center, especially under oxidative stress conditions [Hwang et al., 2009]. For this reason, a proteomic approach using MS was employed to verify if the temporary inactivation observed for this enzyme after 21 days of blood storage was due to an oxidation of this active-site cysteine. To this end, in this set of experiments a thiol blocking agent (N-ethylmaleimide) was used before sample preparation to lock any possible GAPDH redox forms, so as to avoid artifacts of oxidation upon RBC lysis and extract preparation [Murphy 1960, Rinalducci et al., 2011]. Membranes and cytosol of stored RBC were then analyzed by reduced and nonreduced Western blotting using antibodies to GAPDH. Interestingly, under nonreducing conditions, neither dimers nor higher-molecular-weight aggregates of GAPDH were detected throughout the storage, suggesting that storage-induced oxidative stress does not lead to the formation of interdisulfide-linked GAPDH molecules (data not shown). The reactive gel bands

corresponding to GAPDH were excised, subjected to trypsin digestion, and analyzed by peptide sequencing with nano-LC-ESI-qTOF MS/MS. Figure 3 shows the MS/MS spectra of the containing active-site peptide 146IISNASCTTNCLAPLAK162 (859.43 m/z, 2+) as it was identified from the cytosolic GAPDH gel band at 21 days storage. The results indicate the presence of an intramolecular disulfide bridge between Cys-152 and Cys-156, which reasonably accounts for the reversible inactivation of the enzyme registered after 21 days of storage. In agreement, no oxidative modifications were detected in the same peptide when the GAPDH-corresponding bands were excised and analyzed at 0 days (Fig. 3, right panel).

4.4 DISCUSSION AND CONCLUSION

During blood storage, RBCs undergo an exacerbation of oxidative injury due to the rapid exhaustion of glutathione and its related enzymes (such as glutathione reductase and glutathione peroxidase), with consequent accumulation of a number of structural and biochemical changes (referred to as “storage lesions”) [D’Alessandro et al., 2010]. Hypothermic storage also causes significant metabolic alterations that negatively influence enzyme activities, thus contributing to a rapid fall of glycolytic flux [Gevi et al., 2012]. In fact, an increase of early glycolytic intermediates has been measured within the first 2 weeks of storage, with a swift decrease soon afterward [Gevi et al., 2012]. Nevertheless, RBC storage is accompanied by the constant accumulation of lactate, the end product of anaerobic glycolysis [Gevi et al., 2012]. These observations are therefore suggestive of metabolic fine-tunings, where GAPDH may have a crucial role. This enzyme is recognized as an intriguing example of redox-regulated proteins that undergo reversible oxidation of key cysteine residues to regulate their activity in response to oxidants. Indeed, it functions as a homotetramer where each subunit possesses one redox active cysteine in its catalytic center (Cys-152 in human), which is essential for the initial nucleophilic attack on the aldehyde of GAP [Brandes et al., 2009]. Under oxidative stress, this active-site cysteine has been shown to rapidly become the target of a variety of oxidative thiol modifications (disulfide bond formation, sulfinic and sulfonic acids, S-thiolation, S-nitrosylation) by different reactive oxygen and nitrogen oxide species [Brandes et al., 2009, Hwang et al., 2009]. Remarkably, our findings demonstrated an evident decline in the cytosolic activity of

GAPDH at approximately 21 days of storage. As is known, GAPDH catalyzes the reversible oxidative phosphorylation of GAP to 1,3-DPG in the presence of NAD⁺ and inorganic phosphate. Therefore, the observed trend of enzymatic activity is consistent with the previously detected rapid consumption of GAP occurring between 7 and 21 days of storage [Gevi et al., 2012]. Inactivation of GEs has been long attributed to their selective binding to CDB3 [Chu et al., 2008]; however, we found that membranes of 21-day-stored RBCs contain few bound subunits of GAPDH (see Fig. 2B). On the whole, these results indicated a storage-dependent impairment of the GAPDH activity that did not correlate with the revealed concentration of the enzyme in the cytosol, thus favoring the hypothesis of a reversible inactivation due to the oxidation of thiol groups at the catalytic site. In support of this, we identified for the first time the storage-dependent formation of an intramolecular disulfide bridge between the catalytic Cys-152 and Cys-156 of cytosolic GAPDH, which was present in the containing active-site peptide only at 21 days of storage. This in agreement with previous observations demonstrating that oxidative stress-induced reactive oxygen species (probably in the form of hydroxyl radicals and superoxide generated through HaberWeiss and Fenton reactions from Hb heme iron) accumulated over the first 3 weeks of storage, to reach a plateau at 21 days [D'Alessandro et al., 2012]. Interestingly, reversible oxidation of the GAPDH catalytic Cys-152 has been associated with a temporary inactivation of the enzyme that uses this posttranslational modification to reroute metabolites from glycolysis to PPP, thereby increasing the cell capacity to defend against oxidative stress [Brandes et al., 2009, Hwang et al., 2009]. Accordingly, NADPH formation continued to rise during storage, as to reach doubled levels at 42 days in comparison to 0-day control [D'Alessandro et al., 2012]. However, the ratio of metabolic intermediates of the PPP and glycolysis^{9,22} indicates that such a defensive mechanism might only be transitory, resulting in a metabolic lesion leading to the progressive impairment of oxygen-dependent metabolic modulation in stored RBCs [Messana et al., 2000]. Very interestingly, cytosolic GAPDH activity is partially restored (to near 0-day levels) toward the end of storage. Both glutaredoxin and thioredoxin systems are known to reverse oxidative thiol modifications on proteins [Holmgren et al., 2005], thus regenerating enzyme activities from inactivation due to disulfide formation. However, direct evidence of their involvement in stored RBCs is missing so far. What is certain is that glycolysis does not

stop (in agreement with the accumulation of glycolytic end products) [Gevi et al., 2012, D'Alessandro et al., 2012]. or at least it is switched to producing metabolites involved in antioxidant responses. Still, it should be noted that between 28 and 42 days a large amount of GAPDH was found in its membrane-state. Since GAPDH is an abundant cellular protein, it is possible that a subpopulation of the enzyme undergoes irreversible modifications, so as to be localized at the membrane level for subsequent elimination by microvesiculation. Such an observation (i.e., this storage-related late membrane recruitment of GAPDH) deserves more consideration in the light of the fact that a significant increase in tyrosine phosphorylation of Band 3 was observed between the fourth and fifth weeks of storage by Antonelou and colleagues [Antonelou et al., 2012] in SAGM preserved RBCs. As this phosphorylative event at the NH₂ terminus of Band 3 is known to displace GEs (including GAPDH),¹ it is reasonable to suppose that additional docking sites on the RBC membrane may be involved in the GAPDH attachment that we observed from 28 days onward. This assumption is consistent with the recent findings of Puchulu-Campanella and colleagues [Puchulu-Campanella et al., 2013] showing the existence of secondary binding sites for GEs on RBC membranes in correspondence not only of Band 3, but also α - and β -spectrin, ankyrin, actin, p55, and Protein 4.2. In conclusion, RBC GAPDH may play a main role in transitory redirection of metabolites to PPP to augment NADPH production, both *in vivo* and during storage. To our knowledge, this finding is the first evidence for a GAPDH thiol-based redox switch in human RBCs, whose regulation may represent a fundamental strategy to fine-tune cellular metabolism and presumably signal transduction.

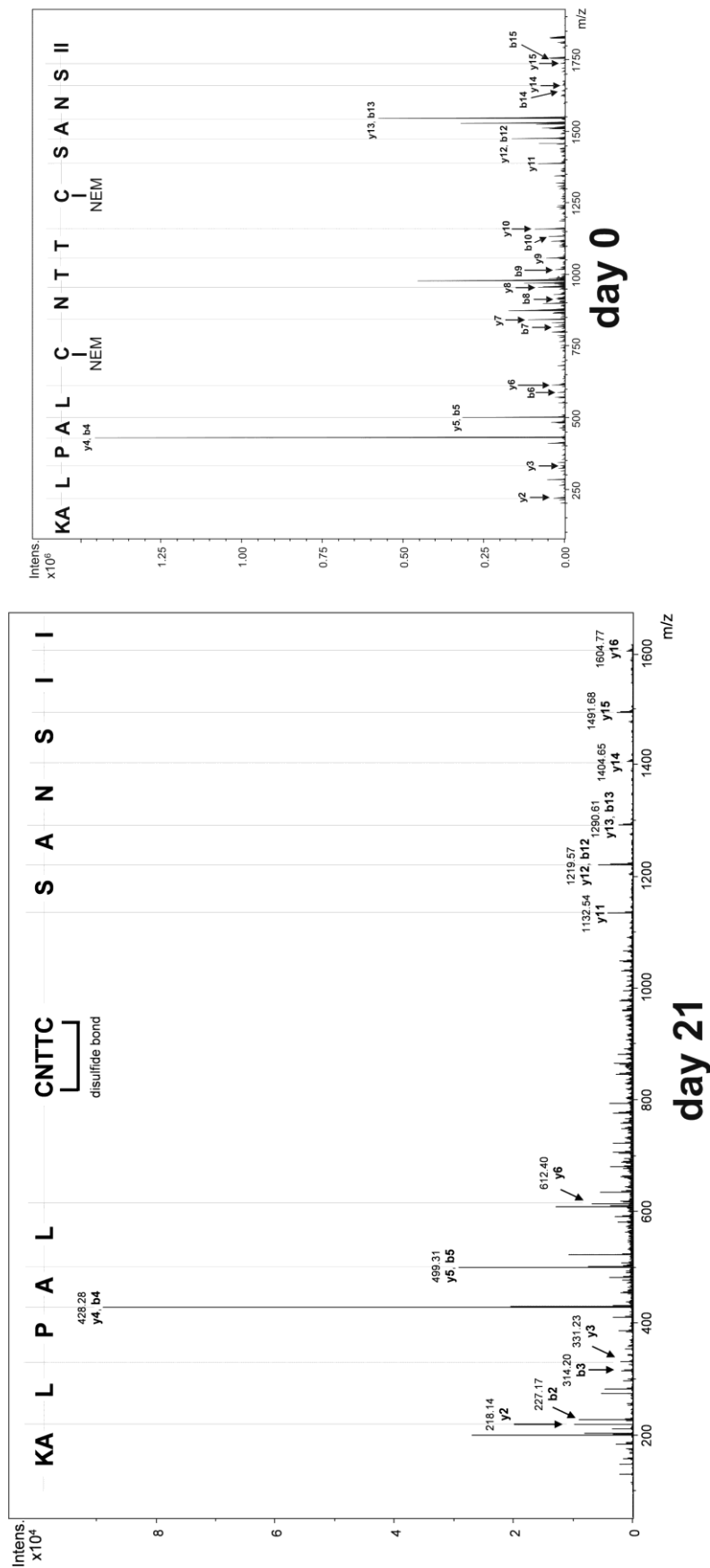


Figure 3—Oxidative modification in the catalytic site of cytosolic GAPDH as identified by nano-LC-ESI Q-TOF MS/MS. (Left) MS/MS spectrum of the active-site tryptic peptide ¹⁴⁶IISNASCNTNCLAPLAK¹⁶² (859.43 m/z, 2+) as detected from 21-day-stored RBCs. Analysis of the y-type sequence ions indicated an intramolecular disulfide bridge between Cys-152 and Cys-156. As expected, disulfide cross-linking dramatically reduces the intensity of produced fragment ions corresponding to the peptide sequence stretch in between (i.e., ¹⁵²CTTNC¹⁵⁶). Moreover, due to disulfide formation, the peptide calculated mass was 1716.85 Da, which represents -2 Da of the original unmodified peptide mass. (Right) MS/MS spectrum of the active-site tryptic peptide ¹⁴⁶IISNASCNTNCLAPLAK¹⁶² (985.96 m/z, 2+) as detected from freshly drawn RBCs. As shown, reduced sulphydryl groups of Cys-152 and Cys-156 are trapped by NEM (N-ethylmaleimide, +215 Da).

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CHAPTER 5:
RED BLOOD CELL LIPIDOMICS ANALYSIS THROUGH HPLC-ESI-QTOF:
APPLICATION TO RED BLOOD CELL STORAGE

5.1 Aim of the study

Lipidomics is the systematic identification of the lipid molecular species of a biological matrix (either a cell, organelle, globule, or whole organism) with emphasis on the relative quantitation of composition changes in response to a perturbation, such as ageing or drug treatments [Brown 2012]. While the term “lipidomics” dates back to a decade ago, investigations of the lipid content of specific biological matrices was an already consolidated field of research over the last fifty years [Philips et al., 1959]. In particular, this holds true for those matrices that are largely available and display limited biological complexity, such as anucleated cells and, in particular RBCs [Philips et al., 1959, Novgorodtseva et al., 2011]. Indeed, RBCs are also devoid of organelles and of any *de novo* lipid synthesis capacity, which makes their lipidome rather stable in comparison to other cell types. Indeed, phospholipid synthesis is known to be active in reticulocytes and suppressed in mature RBCs [Percy et al., 1973]. Nonetheless, alteration of lipid homeostasis is strictly tied to membrane reorganization during RBC ageing *in vivo* and *in vitro* (RBC storage), mainly owing to lipid peroxidation phenomena which promote membrane shape alterations through the progressive loss of lipids (and membrane-associated proteins) via vesiculation [D'Alessandro et al., 2012, Dinkla et al., 2012]. Therefore, it is small wonder that the RBC lipidome has long attracted a great deal of interest over the last five decades. Yet in 1959, Phillips and Roome provided a preliminary portrait of the human RBC phospholipidome [Philips et al., 1959]. However, it was only in 1960 that Hanahan and colleagues described a more complex scenario, also by including species-specific differences between human and bovine RBCs [Hanahan et al., 1960]. Four years later, Ways and Hanahan reported a detailed lipid class composition of normal human RBCs, indicating the following percentages: cholesterol 25%, choline glycerophosphatides 30%, sphingomyelin 24%, ethanolamine glycerophosphatides 26%, and serine glycerophosphatides 15% [Ways et al., 1964]. Meanwhile, Farquhar and Ahrens [Farquhar et al., 1963] had showed that 67% of the PE, 8% of the PS, and 10% of the lecithin of human RBCs are in the plasmalogen form, with a vinyl ether linkage at the sn-1 and an ester linkage at the sn-2 position. In 1967, Dodge and Philips described a silicic acid thin-layer chromatography strategy to investigate the phospholipid and phospholipid fatty acids and aldehydes in human RBCs [Dodge et al., 1967]. Thirtythree fatty acids and five aldehydes were

separated and tentatively classified into lipid classes, including phosphatidyl ethanolamine (PE), phosphatidyl serine (PS), lecithin, and sphingomyelin (SM) 24:0 and 24:1, while fatty acid moieties were tentatively attributed. Of note, the values reported by Dodge and Philips [Dodge et al., 1967] were consistent with those by Ways and Hanahan [Ways et al., 1964]. Interesting results were obtained also as far as it concerned the composition in fatty acid moieties of the different lipid classes. About 37% of the total fatty acid in PS was 18:0, while only about 3% was 16:0; in PE and lecithin, 16:0 was the major saturated fatty acid, with the level in lecithin being over twice that in PE. The relative amount of 18:1 was also much lower in PS than in PE and lecithin. The fatty acid distribution of sphingomyelin differed markedly from that of the glycerophospholipids (GP), in particular in the greater degree of saturation [Dodge et al., 1967]. Only about 33% of the fatty acids were unsaturated; in addition, less than 6% of the fatty acids appeared to have more than one double bond and less than 3% more than two double bonds. The 16:0, 24:0, and 24:1 made up almost 7% of the total fatty acids. Essentially all of the 24:0 and most of the 24:1 of the human RBC phospholipids appeared to reside in sphingomyelin. Different instrumentations and techniques have been tested for the improvement of lipid analysis. During the last two decades, big technological strides have prompted the dissemination of chromatography separation and mass spectrometry-based lipidomics studies of RBCs [Han et al., 1994, Novgorodtseva et al., 2011]. At the dawn of the mass spectrometry-based lipidomics era the complexity of the lipidome did not enable comprehensive studies like the ones performed with thin layer chromatography (TLC) or gas chromatography (GC) described in the previous paragraphs [Philips et al., 1959, Dougherty et al., 1987]. The expensive instrumentation and the lack of bioinformatic tools to handle the high-throughput amount of data collected via the mass spectrometry-based workflow hampered at first its diffusion in the field [Brown 2012]. More recently, the introduction of highly accurate and less expensive instruments (in comparison to the ones available decades ago) was also paralleled by consistent improvements in the field of bioinformatic elaboration of the raw mass spectra [Brown 2012]. The acquired expertise have helped laboratories worldwide to cope with the intrinsic difficulties related to lipid mass attribution and fueled new efforts to bring about the systematic classification of lipid species and structures [Fahy et al., 2005, Sud et al., 2007]. The current burgeoning

of OMICS disciplines has thus given new verve to the field of lipidomics research, while enabling further steps forward. Regarding RBC lipid homeostasis, as premised by Farquhar and Ahrens [Farquhar et al., 1963], lipid composition of human RBCs is largely influenced by the diet. In this view, Dougherty and colleagues performed an extensive investigation to relate region specific diets to the lipid content of plasma, platelets and RBCs [Dougherty et al., 1987]. By comparing RBCs of individuals from rural areas in Finland, Italy (province of Viterbo) and the United States, the Authors demonstrated how diets largely relying on fish and olive oil consumption (in Finland and Italy, respectively), resulted in a significant decrease (in comparison to the US counterparts) in the levels of polyunsaturated fatty acids (PUFA), which they relate to the potential production of unhealthy prostaglandins (thromboxane and prostacyclins) byproducts [Dougherty et al., 1987]. Finally, the Authors also noted that in all plasma and RBC glycerolphospholipids, the monounsaturated fatty acids (especially oleic acid 16:1 and palmitic acid, 16:0) were highest in the Italian and the saturated fatty acids were highest in the Finnish samples. In this frame, we exploit novel databases such as LIPID MAPS and ad hoc software suites for mass spectrometry-based metabolomics analyses (such as MAVEN [Clasquin et al., 2012]) to address the key biological issue of the RBC lipidome. Our investigation shares some features with the study by Dougherty and colleagues [Dougherty et al., 1987], for it was performed on RBCs collected from subjects living in the province of Viterbo, where olive oil consumption represents a central aspect of the diet. We further address the RBC storage issue (from a lipidomic standpoint) as to conclude that wider transfusion medicine-relevant studies should be carried out to investigate whether inter-regional donor differences might lie upon peculiar RBC lipidomic profiles, which in turn are likely to reflect the heterogeneity of local alimentation regimes across Italy.

5.2 Materials and Method

5.2.1 Sample collection

Red blood cell units were drawn from healthy donor volunteers according to the policy of the Italian National Blood Centre guidelines (Blood Transfusion Service for donated blood) and upon informed consent in accordance with the declaration of Helsinki. We

studied RBC units collected from 10 healthy donor volunteers [male=5, female=5, age 39.4 ± 7.5 (mean $\pm$ S.D.)]. RBC units were stored for up to 42 days under standard conditions (CDP-SAGM, 4°C), while samples were removed aseptically for the analysis on a weekly basis (at 0, 7, 14, 21, 28, 35 and 42 days of storage).

5.2.2 Untargeted Metabolomics Analyses

5.2.2.1 Metabolite extraction

For each sample, 0.5mL from the pooled erythrocyte stock was transferred into a microcentrifuge tube (Eppendorf ® Germany). Erythrocyte samples were then centrifuged at 1000g for 2 minutes at 4°C. Tubes were then placed on ice while supernatants were carefully aspirated, paying attention not to remove any erythrocyte at the interface. Samples were extracted following the protocol by D'Alessandro et al. [D'Alessandro et al., 2011]. The erythrocytes were resuspended in 0.15mL of ice cold ultra-pure water (18 M Ω) to lyse cell, then the tubes were plunged into a water bath at 37°C for 0.5 min. Samples were mixed with 0.6mL of -20°C methanol and then with 0.45mL chloroform. Subsequently, 0.15mL of ice cold ultra-pure water were added to each tube and they were transferred to - 20°C freezer for 2-8 h. An equivalent volume of acetonitrile was added to the tube and transferred to refrigerator (4°C) for 20 min. Samples with precipitated proteins were thus centrifuged for 10000 x g for 10 min at 4 °C . Finally, samples were dried in a rotational vacuum concentrator (RVC 2-18 - Christ GmbH; Osterode am Harz, Germany) and re-suspended in 200 μ L of water, 5% formic acid and transferred to glass auto-sampler vials for LC/MS analysis.

5.2.3 Rapid Resolution Reversed-Phase HPLC

An Ultimate 3000 Rapid Resolution HPLC system (LC Packings, DIONEX, Sunnyvale, USA) was used to perform metabolite separation. The system featured a binary pump and vacuum degasser, well-plate autosampler with a six-port micro-switching valve, a thermostated column compartment. Samples were loaded onto a Reprosil C18 column (2.0mm $\times$ 150mm, 2.5 μ m - Dr Maisch, Germany) for metabolite separation. For lipids multi-step gradient program was used. It started with 8% solvent A (ddH₂O, 20 mmol L⁻¹ ammonium formate; pH 5) to 6% solvent A for 3 min then to 2% solvent A for 35

min and finally to 100% solvent B (methanol) in 30 minutes. At the end of gradient, the column was reconditioned with 8% solvent A for 10 min. The overall run time was 68 min. Column oven was set to 50°C and the flow rate was 0.2 mL/min.

5.2.4 Mass spectrometry analysis through microTOF-Q

Due to the use of linear ion counting for direct comparisons against naturally expected isotopic ratios, time-of-flight instruments are most often the best choice for molecular formula determination. Thus, mass spectrometry analysis was carried out on an electrospray hybrid quadrupole time of flight mass spectrometer MicroTOF-Q (Bruker-Daltonik, Bremen, Germany) equipped with an ESI-ion source. MS analysis was carried out in negative ion mode capillary voltage 2800V, nebulizer 45 psi and dry gas of 9 l/min, scan mode 100-1500 m/z. For sample injection, solutions were evaporated to dryness and reconstituted in an adequate volume of methanol:ethanol 1:1. Lipids extracts were prepared by dilution to a concentration of 5 pmol*L⁻¹ (where total phospholipids concentration was 2.5 pmol*L⁻¹). Tandem mass spectrometry (MS/MS) is used for glycerophospholipid species structural characterization. Unambiguous species identification is done by analysis of the retention time and fragmentation pattern and through direct comparison against the same parameters, as acquired from chemically defined standards (Avanti Polar Lipids, Inc., Alabaster, Al.), in agreement with Ivanova et al. [Ivanova et al., 2010]. Automatic isolation and fragmentation (AutoMSn mode) was performed on the 4 most intense ions simultaneously throughout the whole scanning period (30 min per run). Calibration of the mass analyzer is essential in order to maintain an high level of mass accuracy. Instrument calibration was performed externally every day with a sodium formate solution consisting of 10 mM sodium hydroxide in 50% isopropanol, water, 0.1 % formic acid. Automated internal mass scale calibration was performed through direct automated injection of the calibration solution at the beginning and at the end of each run by a 6-port divert-valve.

5.2.5 Data elaboration and statistical analysis

In order to reduce the number of possible hits in molecular formula generation, we exploited the in house SmartFormula application of MAVEN [Clasquin et al., 2012], which directly calculates molecular formulae based upon the MS spectrum (isotopic

patterns) and transition fingerprints (fragmentation patterns). This software generates a confidence-based list of chemical formulae on the basis of the precursor ions and all fragment ions, and the significance of their deviations to the predicted intact mass and fragmentation pattern (within a predefined window range of 5 ppm). Triplicate runs for each one of the 10 biological replicate at day 0 and over storage duration were exported as mzXML files and processed through METLIN/XCMS data analysis software (Scripps Centre for Metabolomics) [Tautenhahn et al., 2012] and MAVEN [Clasquin et al., 2012]. Mass spectrometry chromatograms were elaborated for peak alignment (m/z width = 0.025, minfrac = 0.5, bw = 5), matching and comparison of parent and fragment ions, and tentative metabolite identification (within a 20 ppm mass deviation range between observed and expected results against the imported LIPID MAPS database [Sud et al., 2007] – annotations included adduct ions in positive ion mode). XCMS and MAVEN are open-source software that could be freely used or downloaded from their websites (<http://metlin.scripps.edu/download/> and <http://genomicspubs.princeton.edu/mzroll/index.php?show=download>). Quantitative variations were determined against day 0 controls and only statistically significant results were considered (fold change > 2.5 and ANOVA p -values < 0.01). Data were further refined and plotted with GraphPad Prism 5.0 (GraphPad Software Inc.).

5.3 Results and Discussion

HPLC-MS analysis of the RBC lipidome yielded the tentative identification of a huge number of lipid molecules on the basis of accurate intact mass values and retention times (RT). Results were further validated against MS/MS feature transitions (fragmentation patterns) for RBC storage time course analyses, where we reported statistically significant variations ($p < 0.01$ ANOVA) of specific lipid molecules over storage duration on a weekly basis in comparison to day 0 controls (Table 1). This helped coping with the difficulties related to the attribution of fatty acid moieties in detected lipids, a problem that hampered major translational applications of early MS-based approaches to the RBC lipidome [Han et al., 1994, Beermann et al., 2005]. A 2D map overview of the lipid features identified in a single run is provided in Figure 1, where compound class specific separations are indicated according to the established nomenclature (fatty acids – FA; glycerolipids – GL; glycerophospholipids – GP;

sphingolipids – SP; sterols – ST; prenols – PR and polyketides – PK). While FA, GL, GP and SP eluted rather early (within the first six minutes of RT), ST first and PR or PK displayed higher RTs, consistently with their more hydrophobic nature. In the following paragraphs, we will detail the major findings of the currently proposed investigation through the description of the main distinct lipid classes. Results will be discussed in the light of existing literature in the field.

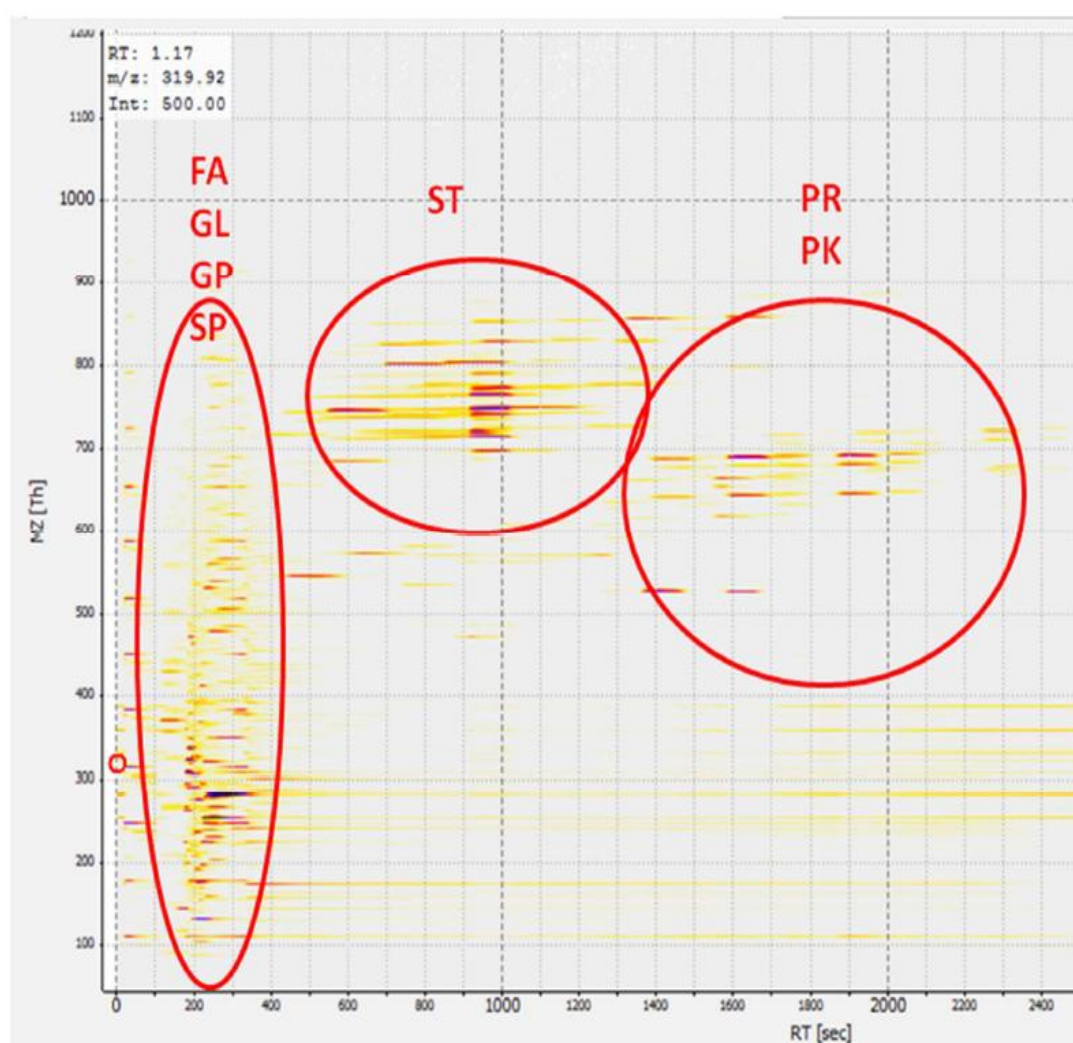


Figure 1 2D map overview of the lipid features identified in a single run compound. Class specific separations are indicated according to the established nomenclature (fatty acids – FA; glycerolipids – GL; glycerophospholipids – GP; sphingolipids – SP; sterols – ST; prenols – PR and polyketides – PK).

Table 1. Time course alterations of lipids in long stored Red Blood Cells (RBCs). The table reports a weekly comparison of the lipidomes of stored RBCs. Mass spectra chromatograms of day 0, 7, 14, 21, 28, 35 and 42 lipid extract samples were aligned and matched with XCMS software. Statistically significant variations (fold change > 2.5 and p-value < 0.01 ANOVA) against day 0 controls were reported in the table, depending on the specific lipid class (through a direct reference to the LIPIDMAPS database). Red=Increase;

Lipid class	0 vs 7	0 vs 14	0 vs 21	0 vs 28	0 vs 35	0 vs 42
FA	Anandamide (20:1, n-9)	Clupanodonyl carnitine	Cervonyl carnitine		Maylene-18	11,12-dihydroxy arachidic acid
	4-fumarylacetoacetic acid		Dodecanoyl carnitine		N-(5-hydroxy-pentyl) arachidonoyl amine	omega-hydroxy hendecanoic acid
	3-O-L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoic acid		triacontan-1,14-diol		Triacontan-1,14-diol	15-oxo-18Z-tetracosenoic acid
	1-O-[(6'-O-hexadecanoyl)-aD-glucopyranosyl]-(2-hexadecanoyloxy)-eicosan-1-ol		Hentriacontan-16-one		Hentriacontan-16-one	
			15(S)-15-methyl PGF2a isopropyl ester		(Z)-N-(2-hydroxyethyl) hexacos-17-enamide	
			PGF2a dimethyl amine		Prostaglandin E2	
			Prostaglandin E2 (1R,2R)-3-oxo-2-pentyl-cyclopentanehexanoic acid		3-methyl-tetradeconoic acid	
	C24:1-OH Sulfatide		7-oxo-11E-Tetradecenoic acid		5,13-docosadienoic acid	
	DG(17:1(9Z)/17:1(9Z)/00)					
	54:3 SLBP A					
DG						
TG	TG(20:5 (5Z,8Z,11Z,14Z,17Z)/22:3 (10Z,13Z,16Z)/22:5 (7Z,10Z,13Z,16Z,19Z)) [iso6]					
	TG(17:0/18:2 (9Z,12Z)/20:0) [iso6]					
P				PC(O-1:0/O-18:0)		PC(10:0/18:0)
C						
GP	PIP2(16:0/18:0)					
	D-myo-Inositol-1,2,4,5,6-pentaphosphate	D-myo-Inositol-1,2,4,5,6-pentaphosphate			PI(13:0/0:0)	D-myo-Inositol-1,3,4-triphosphate (sodium salt)
	Inosine 5'-tetraphosphate					
P						
E			LysolPE(0:0/22:2 (13Z,16Z))		LysolPE(0:0/22:2 (13Z,16Z))	

Table 1 Continuation) Red=Increase; Blue=decrease

Lipid class	0 vs 7	0 vs 14	0 vs 21	0 vs 28	0 vs 35	0 vs 42
ST	1-Hydroxyvitamin D3 3-D-glucopyranoside	Vitamin D3 / Cholecalciferol (Vitamin D3) skeleton	1 α -hydroxy-25-methoxyvitamin D3 / 1 α -hydroxy-25-methoxycholecalciferol	Cucurbitacin H	Cucurbitacin H 1-Hydroxyvitamin D3 cellobioside 19-norandrosterone	(6R)-vitamin D2 6,19-sulfur dioxide adduct / (6R)-ergocalciferol 6,19-sulfur dioxide adduct
		5 β -Cholestane-3 α ,7 α -diol	Campesterol	Desmosterol (d6)	Campesterol 5 α -Tetrahydrocorticosterone Desmosterol (d6)	
			1 α -hydroxy-23-[3-(1-hydroxy-1-methylethyl)phenyl]-22,22,23,23-tetrahydro-24,25,26,27-tetanorvitamin D3	(22R)-1 α ,25-dihydroxy-22-methoxy-26,27-dimethyl-23,23,24,24-tetradehydrovitamin D3 / (22R)-1 α ,25-dihydroxy-22-methoxy-26,27-dimethyl-23,23,24,24-tetradehydrocholecalciferol	(2-fluoroethyl)-25-hydroxy-26,27-dimethyl-24 α -homofluoroethyl)-25-hydroxy-26,27-dimethyl-24 α -homofluoroethyl)-25-hydroxy-22-oxacholecalciferol 3 α ,7 α ,12 α ,24-tetrahydroxy-24-methyl-5 α -cholestan-26-olc acid	
	1 α ,25-dihydroxy-11-(4-hydroxymethylphenyl)-9,11-didehydrovitamin D3 / 1 α ,25-dihydroxy-11-(4-hydroxymethylphenyl)-didehydrocholecalciferol	1 α -hydroxy-2 β -(5-hydroxypentoxo)vitamin D3 / 1 α -hydroxy-2 β -(5-hydroxypentoxo)cholecalciferol	1 α ,25-dihydroxy-11-(4-hydroxymethylphenyl)-9,11-didehydrovitamin D3 / 1 α ,25-dihydroxy-11-(4-hydroxymethylphenyl)-didehydrocholecalciferol		1 α -hydroxy-22-(3-hydroxyphenyl)-23,24,25,26,27-pentanorvitamin D3 / 1 α -hydroxy-22-(3-hydroxyphenyl)-23,24,25,26,27-pentanorcholecalciferol	
		1 α -hydroxy-25-methoxyvitamin D3 / 1 α -hydroxy-25-methoxycholecalciferol	3 α ,12 α ,25-trihydroxy-5 β -cholestan-7-one	3 α ,12 α ,25-trihydroxy-5 α -cholestan-7-one		
			(22R)-1 α ,25-dihydroxy-22-methoxy-26,27-dimethyl-23,23,24,24-tetradehydrovitamin D3 / (22R)-1 α ,25-dihydroxy-22-methoxy-26,27-dimethyl-23,23,24,24-tetradehydrocholecalciferol		Gorgosterol 57-Cholestane-3 β ,7 β -diol	
					Ecalcidene	

Table 1 Continuation) Red=Increase; Blue=decrease

Lipid class	0 vs 7	0 vs 14	0 vs 21	0 vs 28	0 vs 35	0 vs 42
SAC	1-(O-alpha-D-glucopyranosyl)-3-keto-octacosanetriol			1-O-alpha-D-glucopyranosyl-1,2-nonadecandiol		
PR			30,32-dihydroxy-2b-methyl-bishomohopane (+)-24-Dammarene-3alpha,12beta,20S-triol 2-Hexaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone	4,4'-Diapo-zeta-carotene	4,4'-Diapo-zeta-carotene 2-Hexaprenyl-3-methyl-6-methoxy-1,4-benzoquinone 3-Hexaprenyl-4-hydroxy-5-methoxybenzoic acid 4,4'-Diapophytoene	
PK			Delphinidin 3-(6-p-coumaroylglucoside)-5-[6-(malonyl)-4-(rhamnosyl)glucoside]]			
Cer			C-8 Ceramide		C-8 Ceramide	C-2 Ceramide
SP			Ceramide (d18:1/12:0)		Ceramide (d18:1/12:0)	
			N,N'-trimethyl-sphingosine	Sphingosine	Phytosphingosine	
				Phytosphingosine	D-erythro-Sphingosine C-15	

5.3.1 Fatty acids

Fatty acid distribution indicated that palmitic acid (FA C16:0 – Figure 2) was the most abundant free fatty acid. This is also consistent with oldest reports on the RBC fatty acid composition available from the literature [Ways et al., 1964], despite the extreme differences between the TLC and the HPLC-MS analytical approaches. Palmitic acid might be tied to the modulation of calcium signaling in RBCs by mediating Ca^{2+} fluxes via specific membrane pores [Belosludtsev et al., 2010], thereby modulating RBC survival. Among the most abundant individual fatty acids we could detect 16:0 (palmitic), 18:0 (stearic), 18:1 (oleic), and 22:6 n-3 (docosahexaenoic acid), in agreement with previous studies on fatty acids of erythrocytes obtained from healthy Italian subjects [Pala et al., 2001].

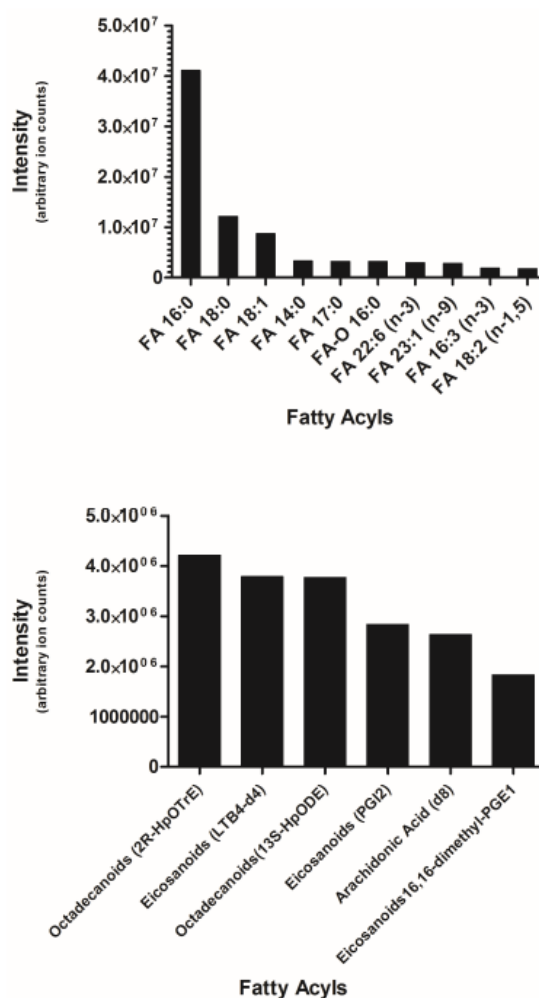


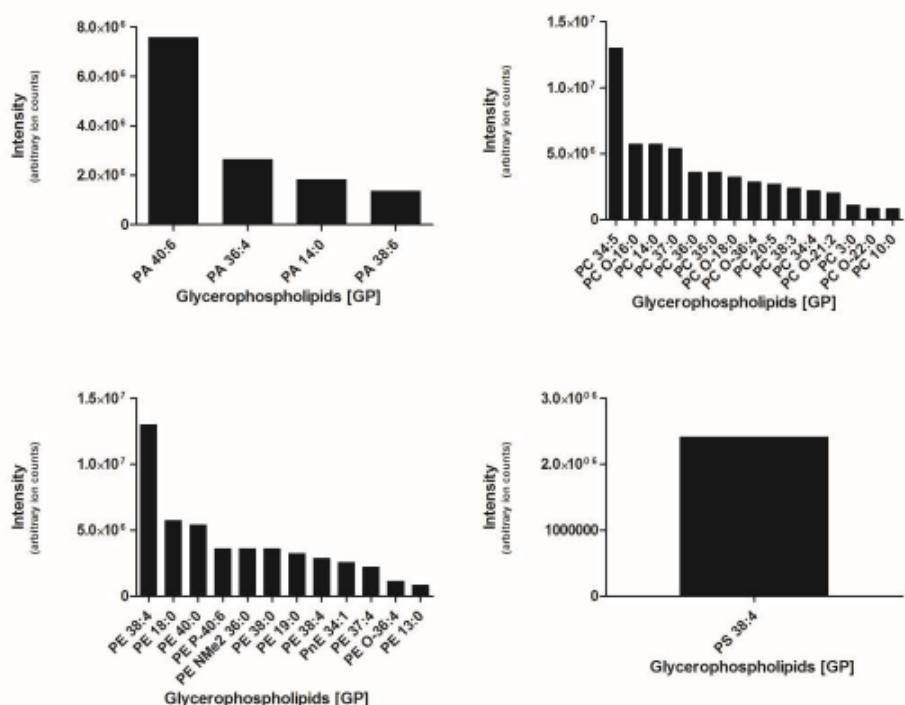
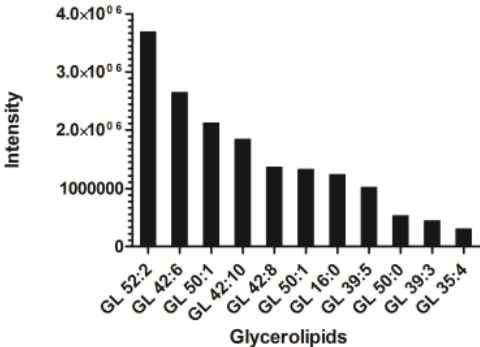
Figure 2-Fatty acid distribution obtained by exporting data from microQtof as mzXML files and processed through MAVEN by interrogating LIPID MAPS database.

Furthermore, octadecadienoic acid (18:2 n- 1,5) had already been reported among the most abundant ten fatty acids of RBCs [Ways et al., 1964]. The abundance of oleic acid in particular was an expected result, since olive oil holds a key role in the frame of the Mediterranean diet and, in particular, in the province of Viterbo (Italy) where blood samples were collected from healthy donor volunteers. The intertwinement between oleic acid relative concentrations and high olive oil consumption rates had already been postulated and demonstrated through TLC approaches [Dougherty et al., 1987], and hereby confirmed through MS. On the other hand, no previous investigation indicated myristic acid (14:0) as one of the most abundant fatty acid in RBCs, except for those studies suggesting a role for myristic acid supplementation as a substitute of oleic acid in the diet, which results in the relative increase of α -linolenic and doxosaheptaenoic acid levels [Rioux et al., 2008] and alterations of RBC membrane fluidity [Dabadie et al., 2006]. Analogously, heptadecanoic acid (17:0) has been proposed as a controversial biomarker for the assessment of energy and macronutrient composition in response to specific diets [29]. Eicosanoids and octadecanoids and their peroxidation products (relative abundances are reported in Figure 1) are thought to play a role in mediating RBC maturation from reticulocytes by promoting the degradation of mitochondrial membranes and thus elimination of these organelles [Grüllich et al., 2001]. Also, eicosanoids serve as substrates for cyclooxygenase, lipoxygenase and epoxygenase activities, which result in the production of pro-inflammatory factors that are associated with increased cardiovascular risk and cancer [Smith et al., 2002].

5.3.2 Glycerolipids and glycerophospholipids

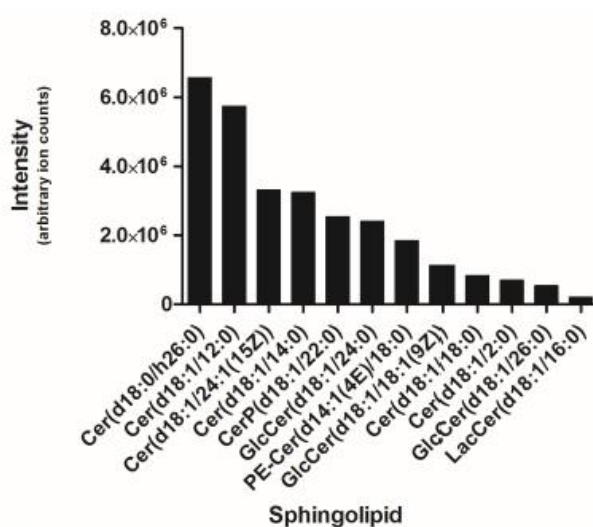
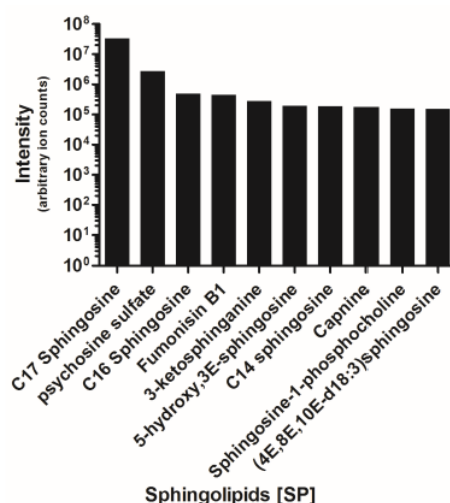
Relative abundances of RBCs glycerolipids (GL) and glycerophospholipids (GP) are reported in Figure 3 and 4, respectively, whereas the latter class has been further subdivided into phosphatidic acid (PA), phosphatidylcholines (PC), phosphatidylethanolamines (PE) and phosphatidylserine (PS), in the light of the observed elevated concentrations in RBCs. Fatty acid incorporation stages into RBC membrane GLs and GPs has been long investigated [Oliveira et al., 1964, Donabedian et al., 1967, Mulder et al., 1962], indicating higher rates for reticulocytes in comparison to adult RBCs [Shohet et al., 1968, Van Gastel et al., 1965]. Indeed, RBC, GL and GP metabolism is a key aspect in RBC survival [Mulder et al., 1965], since during their 120

days approximate lifespan in the circulatory system RBCs shed approximately 1 microvesicle/h, thus continuously remodeling their membrane and its lipid composition. Also, early approaches to GP composition of RBCs have been purported via TLC [Skipski et al., 1964] indicated a relation of GP composition with RBC membrane anomalies, such as in the case of spherocytosis [Kates et al., 1961]. These considerations are relevant in the light of the incomplete long chain fatty acid synthesizing system which characterizes RBCs [Pittman et al., 1966]. The introduction of high-resolution capillary gas chromatography approaches recently shed new light on this delicate issue [Jakobik et al., 2009], further evidencing compositional anomalies of GL and GP in cancer patients [Mikirova et al., 2004]. Our results provide further supporting evidence about elevated levels of C16:0 and C18:0 fatty acids in lyso-PCs from adult RBCs (Figure 4), as previously reported with different approaches [Dougherty et al., 1987, Jakobik et al., 2009, Mikirova et al., 2004]. Analogous considerations can be made for PE 38:4, PE 40:6 and lyso-PE 18:0, as well as for PS 38:4 (Figure 4), in agreement with the literature [Dougherty et al., 1987, Mikirova et al., 2004]. In particular, PS 38:4 had been recently indicated as the most abundant RBC-specific PS, in comparison to other blood cell types [Mikirova et al., 2004]. Distribution of saturated and unsaturated fatty acids in GL and GP was also consistent with the literature [Ways et al., 1964, Dougherty et al., 1987, Mikirova et al., 2004]. As expected, compositional differences were observed as well, which are probably attributable to the different diets of the subjects enrolled in the present study in comparison to data available from literature.



5.3.3 Sphingolipids, sterols and prenol lipids

Sphingolipids (SP), among which ceramides (Cer), have been recently associated with in vivo and in vitro ageing of RBCs [Dinkla et al., 2012]. Though the mechanisms have not yet been fully elucidated, sphingolipids and ceramides seem to affect RBC survival by serving as signaling molecules upon acid sphingomyelinase hydrolysis of sphingomyelin into ceramide [Smith et al., 2008] or rather they directly affect RBC membrane stability by forming specific pores and thus altering membrane permeability and potential [Siskind et al., 2005]. Ceramide-enriched membrane domains have been indeed associated with hot-cold hemolysis [Montes et al., 2008]. Besides, SP metabolites, including ceramides, sphingosine and sphingosine 1-phosphate have recently emerged as a new class of lipid biomodulators also in the extracellular space [Tani et al., 2006]. In Figures 5 and 6 we report the relative abundances of the top SP, with a focus on ceramides, respectively. Of note, C16 sphingosine (Figure 5) has been recently reported to be the most abundant RBC-specific SP [Leidl et al., 2008]. On the other hand, while we expected elevated levels of Cer 24:1 and 24:0, in agreement with Leidl et al. [Leidl et al., 2008], we could instead observe elevated levels of Cer 18:0 and 18:1 in all ceramide subclass (Figure 6), which might reflect the relative composition of free fatty acid, as mentioned in the previous paragraphs. Recent studies have also demonstrated that sphingolipids dynamically cluster with sterols to form lipid microdomains or rafts, which function as platforms for effective signal transduction and protein sorting [Simons et al., 1987]. Sterol profiling of RBCs is also a powerful diagnostic tool to investigate the effects of total parental nutrition diet supplementation to the newborn, a lifesaving therapy in children with intestinal failure [Pianese et al., 2008]. An overview of the most abundant sterol lipids is provided in Figure 7, where sterol lipids are reported with their relative name from the Lipid Maps database [Sud et al., 2007], owing to the impossibility to adapt graphic limitations to the lengthy extended names of each sterol lipid. Prenol lipids are often under-investigated class of lipids, which are synthesized from five carbon isoprene units. Recent lipidomics studies focused on plasma levels of dolichols (a group of α -saturated polyprenols characterized by 14 to 24 isoprene subunits) and ubiquinones (a group of 1,4- benzoquinones modified with 9-10 repeated isoprene units) [Quehenberger et al., 2010]. However, to the best of the Authors' knowledge, little is known about the composition of prenol



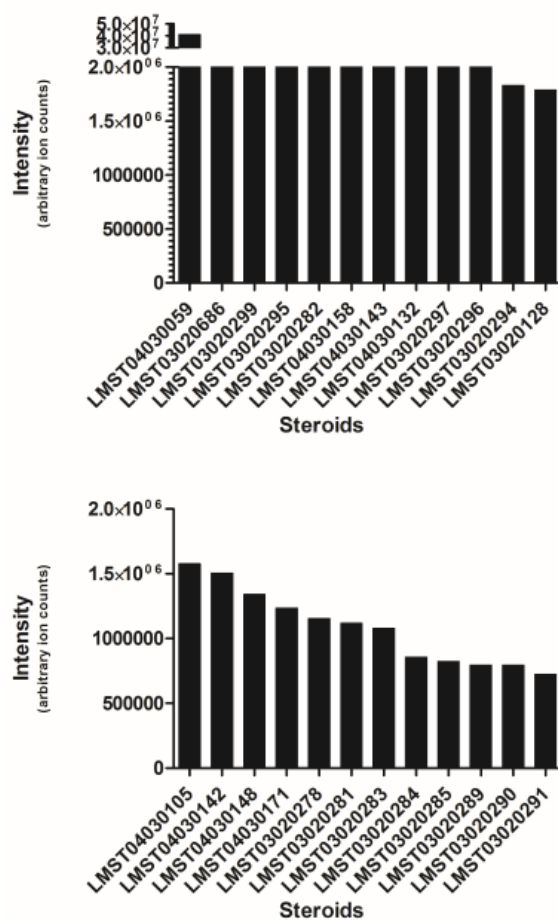


Figure 7-Steroids distribution obtained by exporting data from microQtof as mzXML files and processed through MAVEN by interrogating LIPID MAPS database.

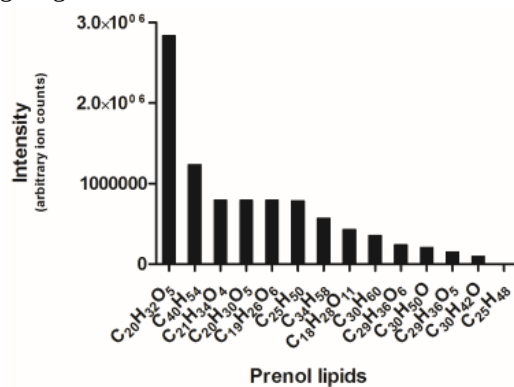


Figure 8-Prenols distribution obtained by exporting data from microQtof as mzXML files and processed through MAVEN by interrogating LIPID MAPS database.

5.3.4 Red blood cell Lipidomics: application to the storage of erythrocyte concentrates

RBC concentrates for transfusion purposes are routinely stored at 2-6°C for up to 42 days, according to international standard guidelines [European Directorate for the

Quality of Medicines & HealthCare 2011]. Despite decades of substantial improvements in the field of RBC storage [D'Alessandro et al., 2012], concerns still arise and persist about the quality of longer stored RBCs, since it is clearly emerging –at least from a biochemical standpoint– that storage progression corresponds to the accumulation of a wide series of RBCs storage lesions [D'Alessandro et al., 2012], as, among others, we could recently document at the morphologic, metabolomics and proteomics level [Blasi et al., 2012, D'Amici et al., 2012, Xu et al., 2010]. On the other hand, lipidomic aspects of RBC storage in the blood bank still lie undisclosed. Recently, Bosman's group demonstrated that specific treatment with exogenous sphingomyelinases resulted in the accumulation of ceramides RBC morphological lesions, thereby mimicking the effects of long-stored RBCs [Dinkla et al., 2012]. Indeed, accumulation of ceramides and their metabolites (sphingosine and sphingosine 1-phosphate – S1P) might promote intrinsic stimuli leading to the exacerbation of ageing phenomena in RBCs [Dinkla et al., 2012], by altering membrane conformation [Siskind et al., 2005] or mediating specific intra- or extra-cellular signaling cascades [Smith et al., 2008]. In this view, it is worthwhile noting that plasma S1P mainly originates from erythrocytes, since RBCs display alkaline (but not acid or neutral) ceramidase activity on D-e-C(18:1)-ceramide [Neidlinger et al., 2006]. First of all, we wish to stress that the most abundant ceramides we could detect in control adult RBCs could be catalogued as C18:0 or 18:1 (Figure 6). Moreover, in the present study prolonged storage of RBC was apparently associated with statistically significant decrease ($p < 0.01$ ANOVA) of ceramides (C-8, Ceramide d18:1/12:0 and ceramide C-2 – Table 1) after three weeks of storage, which is a critical timespan threshold for the accumulation of storage lesions at the biochemical level, as we could previously report at the proteomics and metabolomics level [D'Amici et al., 2012, Xu et al., 2010]. These results are suggestive of a likely ceramidase-mediated digestion of ceramides, or rather of an alteration of the lipid composition of long-stored RBCs probably reflecting the membrane remodeling occurring over RBC storage duration [Blasi et al., 2012]. However, we could also observe a decrease in the levels of several sphingosines (N,N,N-trimethyl sphingosine, sphingosine, phytosphingosine and D-erythro-Sphingosine C-15 – Table 1), which did not help us ruling out any definitive scenario to explain the observed phenomena. After an initial increase (attributable to phospholipase

activities [Chaudhary et al., 2012]), prolonged storage of RBC concentrates hereby resulted in the progressive statistically significant ($p < 0.01$ ANOVA) decrease of a wide series of fatty acids, prostaglandins (such as $\text{PGF}_2\alpha$ and prostaglandin E_2) and fatty acid oxidation products ((1R,2R)-3-oxo-2-pentylcyclopentanehexanoic acid) (Table 1). This is consistent with the reported progressive accumulation of lipid oxidation byproducts in the supernatants of long-stored erythrocyte concentrate units [Gevi et al., 2012, Chaudhary et al., 2012]. The initial increase in the levels of diacyl-glycerols (DG) and triacyl-glycerols (TG) (Table 1) is difficult to interpret, if not in the light of the need for RBCs to cope with the initial free fatty acid accumulation through their sequestering and accumulation in the form of DGs and TGs. This is consistent with the hypothesis that, whether a Save or Sacrifice mechanism is innate in RBCs, as suggested by *in silico* elaborations [Goodman et al., 2007, D'Alessandro et al., 2010], this mechanism is active within the first two weeks of storage [Blasi et al., 2012, D'Amici et al., 2012]. Recently, Bicalho et al. investigated the alterations to the RBCs phospholipidome by performing a direct comparison of fresh RBC phospholipids against the phospholipid composition of RBC-shed microvesicles [Bicalho et al., 2012]. As a result, the Authors could point out the alterations of PS 38:4 and PS 38:1 composition in fresh controls and RBC-derived microvesicles [Bicalho et al., 2012]. In the present study, while we could confirm previous evidences about PS 38:4 being preponderant in RBCs (Figure 4), also in agreement with Leidl et al. [Leidl et al., 2008], we could not detect any statistically significant variation as far as PS are concerned. On the other hand, we could detect significant decrease in the levels of two PCs (O-1:0/O-18:0 and 10:0/18:0 – Table 1), Pes (lyso-PE(0:0/22:2(13Z,16Z))) and lyso-PE(0:0/22:2(13Z,16Z)) – Table 1), while PIs followed a controversial trend, especially within the first two weeks of storage. Finally, sterols, prenols, saccharolipids and polyketides were hereby investigated for the first time within the framework of RBC storage. Intriguingly, all these classes of lipids statistically significant decreases throughout storage duration (Table 1). Most of the observed decreases account for sterols (e.g. desmosterol, gorgosterol), and in particular for vitamin D3-related metabolites (Table 1). This is relevant in the light of the well-established role for Vitamin D in modulating RBC survival [Alexander 1977], also by *in*Yuencing anti-oxidant potential and Ca^{2+} permeability [Holmes et al., 1963], a phenomenon which is strictly tied to erythrocyte-specific apoptosis, also known as

eryptosis [Pompeo et al., 2010]. On the other hand, earliest studies on the likely long term effect of RBC storage on the lipidome suggested that cholesterol loss is limited in comparison to the loss of phospholipids and phosphoinositides [Greenwalt et al., 1990]. Finally, our results about a generalized decrease in lipid contents of the major lipid classes in long stored RBCs also confirm and expand/ complement recent evidences by Acker's group [Bicalho et al., 2013, Almizraq et al., 2013].

5.4 Conclusion

Despite decades of investigations, the field of lipidomics recently drained new lymph from the introduction of recent technical innovations. From TLC to gas chromatography and MS, consolidated lipidomics expertise in the field of RBC biology has paved the way for a deeper understanding of the functioning of this pivotal cell and, in parallel, to the accumulation of a wealth of knowledge that will be soon transferred to the clinical setting. Indeed, owing to their relative abundances and widespread biological activities, lipids are well suited to play the role of biological markers and will soon serve this purpose. In this study, we presented an HPLC-microTOF-Q approach to investigate the RBC lipidome. We could exploit this analytical workflow to consolidate existing knowledge on the RBC lipid composition and individuate statistically significant fluctuations of lipids throughout storage duration of RBC concentrates under blood bank conditions. While this field of research still warrants future investigations, which could be exploiting for example recently introduced imaging mass spectrometry approaches [Mainini et al., 2013], we could indicate ceramides, glycerophospholipids and sterols as key targets of RBC storage lesions to the lipidome, that will deserve further targeted investigations in the future. Finally, in the light of minor differences with other reports available from the literature, we posited how compositional analyses of the RBC lipidome might end up yielding different results on the basis of the background of the blood donor (above all, the diet), which might translate into regionspecific lipidomic alterations over storage progression of RBC concentrates. This is relevant in the light of the constant efforts pursued by transfusion services to improve the quality of blood-derived therapeutics [Liumbruno et al., 2010], by shifting the focus of attention from the end-product (RBC concentrates) to their providers (the donors).

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