Università degli Studi della Tuscia di Viterbo

Dipartimento di Scienze Ecologiche e Biologiche

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Peroxiredoxin-2: Oligomeric characterization of a putative biomarker of oxidative stress in the blood storage scenario

BIO 11

Coordinator Prof. Giorgio Prantera

PhD student Barbara Blasi

Tutor Prof. Lello Zolla

-"Ora ajutami ad uscirne, - disse alla fine Zi' Dima.
Ma quanto larga di pancia, tanto quella giara era stretta di collo.
Zi' Dima, nella rabbia, non ci aveva fatto caso.
Ora, prova e riprova, non trovava più il modo di uscirne.
E il contadino, invece di dargli ajuto, eccolo là, si torceva dalle risa.
Imprigionato, imprigionato lì, nella giara da lui stesso sanata e che ora - non c'era via di mezzo -
per farlo uscire, doveva essere rotta daccapo e per sempre.

"La giara". Luigi Pirandello, 1916.

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Chapter 1

Introduction

1.1 Blood

Blood is an animal connective tissue that is assigned to the transport of substances, nutrients, wastes, and oxygen inside the body. Its color is tipically red, more or less dark relying on its oxygenation *status*. In vertebrates it constitutes the 8-9 % of the total body weight, corresponding to an average volume of 5 liters. Blood pH is used to stay within a very narrow range from 7.35 to 7.45. The maintenance of such values is performed by blood solutes and organs such as lungs and kidneys. The solutes mainly involved in pH control belong to intracellular buffers as phosphate ($H_2PO_4^- \leftrightarrow HP0_4^{2-}$) and carbonate ($HCO_3^- \leftrightarrow H_2CO_3$) buffers. Proteins like carbonic anhydrase are players in these balances through their catalytic activity. Nevertheless, hemoglobin as well contributes to proton management being one of the main blood H^+ acceptor/donor. Finally, lungs and kidneys work by regulation of pO_2 and filtration of electrolytes, respectively.

Blood is certainly the most complex animal tissue, being composed of several kinds of cells (blood corpuscolate component) suspended in plasma. The special fluid-dynamic characteristic of blood allows it to flow through capillary vessels with less resistance than plasma itself.

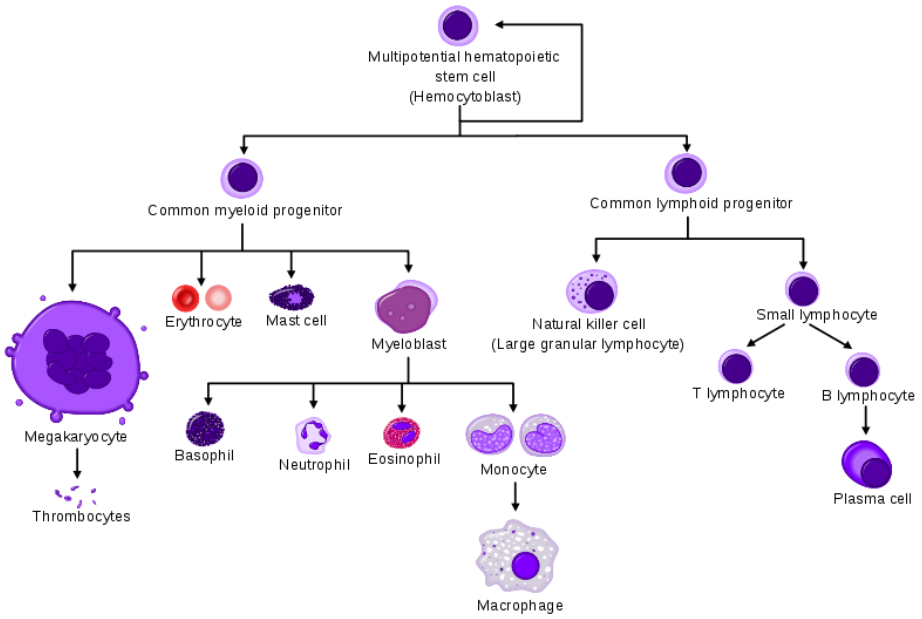

Figure 1. Hemopoiesis.

Thrombocytes, also called platelets, are the cell type involved in hemostasis, the complex process that bring to blood coagulation. Leukocytes, or white blood cells, are responsible of the body defence against viral and bacterial infections. Erythrocytes are the most abundant

blood cells. Their percentage (45 % for men and 40 % for women) is called either Hematocrit (Hct), or packed cell volume (PCV), or erythrocyte volume fraction (EVF) and in mammals is independent from body-size.

1.2 Erythrocytes

Erythrocytes, or red blood cells (RBCs), are anucleated cells with a typical biconcave shape (7µm diameter x 2µm thickness x 90µm^3 of volume) (Fig. 2); in the human species they are around 5000000 in males and 4500000 in females.

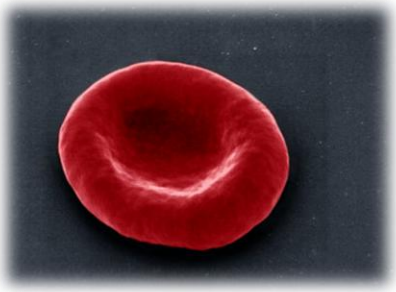

Figure 2. Red blood cell.

The process by which they are produced from the hemocytoblast is called erythropoiesis (from Greek *poiesis* for "production") that in mammals takes place in different organs depending from the life stage of the individual. In particular, in the early stages of fetus, it occurs in the yolk sac, while later on it is moved to spleen and liver. Already in the sixth month after fertilization, the erithropoiesis depends on the bone marrow, that will remain the location for all the rest of life span. Erythropoietin is the hormone responsible for erythropoiesis and it is produced from kidneys in conditions of low O_2 pressure.
In non-pathological conditions, a human red blood cell lives 120 days, 10 of which inside the bone marrow and the remaining in the cardiovascular system. Their removal (hemocatheresis, from Greek *kathairesis* for destruction) occurs in the spleen and, to a lesser extent, in the liver and in the bone marrow itself.

1.2.1 Erythrocyte membrane

The structural organization of the red cell membrane enables it to undergo strong deformation and to maintain the characteristic biconcave shape for all the life span. The membrane is basically made up of lipidic bilayer (about 40 %), with an equal proportion by weight of

cholesterol and phospholipids [Walensky *et al*., 2003], and proteic fraction (about 52 %). Phospholipids show an asymmetrically distribution between the two sides of leaflet; in particular, phosphatidylcholine and sphingomyelin are predominantly located in the outer monolayer, while most phosphatidylethanolamine and all phosphatidylserine (PS), together with the minor phosphoinositide constituents, are confined to the inner monolayer [Mohandas and Gallagher, 2008]. The unequal distribution of lipids has functional role in communication, for instance with immune system. Macrophages recognize erythrocytes that expose PS at the outer surface and so its usual confinement in the inner surface of the membrane is needed to preserve red cells from a premature removal from the cardiovascular system.

More than 100 different proteins compose the red cell membrane and can be classified as integral (transmembrane) and peripheral. The latter are linked to the cytoplasmic surface of the lipid bilayer and constitute the so called cytoskeleton that coats the inner membrane layer of the whole cell (Fig. 3).

Nowadays more than 50 transmembrane proteins has been characterized with around 25 antigen species involved in the blood group definition.

The other kinds of integral proteins exhibit diverse functional heterogeneity, serving as transport proteins, adhesion proteins involved in interactions of red cells with other blood cells and endothelial cells, signaling receptors, and other still undefined activities.

Band 3, one of the most abundant integral proteins, is a glycoprotein. Its transmembrane domain serves as an anion transporter for molecules such as HCO_3^- and Cl^-. The cytoplasmic domain contains the binding sites for several proteins (hemoglobin, protein 4.1, ankyrin and glycolytic enzymes) and it also functions as anchorage of the cytoskeleton to the lipid bilayer. Glicophorins are a group of sialoglycoproteins that, thanks to the carbohydrate residues at the outer surface of the membrane, confer a strong net negative charge to the red cell, preventing them from adhering to the endothelium and aggregating each other. [Bossi and Giardina, 1996]. Other membrane proteins with transport function include aquaporin 1 (water transporter), Glut1 (glucose and L-dehydroascorbic acid transporter), Kidd antigen protein (urea transporter), RhAG (Rh associated glycoprotein, ammonium and carbon dioxide transporter), Na^+-K^+-ATPase, Ca^{++} ATPase, Na^+-K^+-$2Cl^-$ cotransporter, Na^+-Cl^- cotransporter, Na^+-K^+ cotransporter, K^+-Cl^- cotransporter, and Gardos Channel.

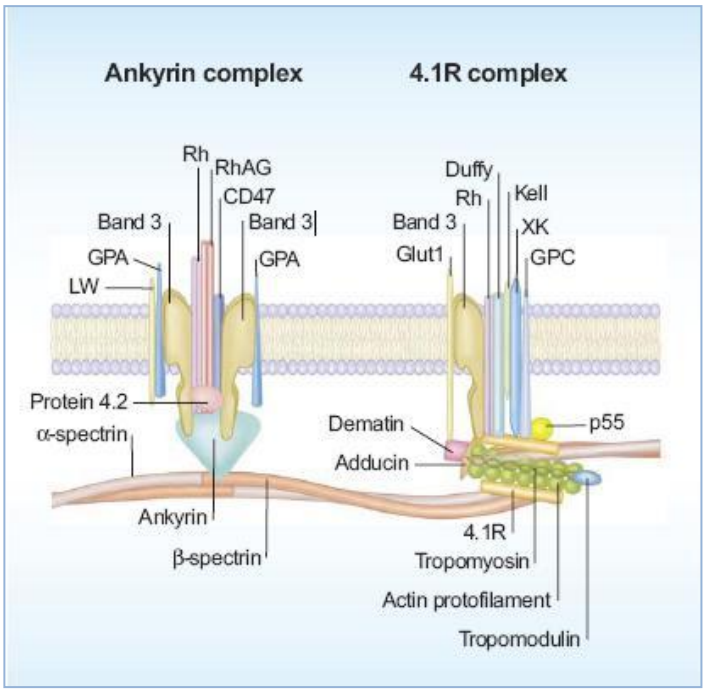

Figure 3. RBC membrane protein organization.

On the other hand, the cytoskeleton proteins are an organized network comprising major (e.g. α- and β-spectrin, actin, protein 4.1, ankyrin) and minor components (protein 4.2, dematin (4.9), α- and β-adducin, tropomyosin, tropomodulin etc.), many of which interact not only with each other, but also with proteins and lipids of the membrane [Pasini *et al*., 2009]. The membrane deformation is possible thanks to modulation of the cytoskeleton structure between extended and compressed form. By the way, the key component of the cytoskeletal network is spectrin, a tetrameric protein ($\alpha_2\beta_2$), the mainly involved in membrane deformability/stability and also in lateral mobility of band 3. α- and β-spectrins form an antiparallel heterodimer and their attachment to the membrane is mediated from two kinds of protein complexes. One is based both on the interaction of spectrin with ankyrin and on this last with band 3 protein while the other on the spectrin interaction with band 4.1, p55 and finally glycophorin C. Thanks to two possible protein conformations, one highly coiled, helical state and one rod-like, spectrin owns spring-like behaviour that confers high flexibility and extensibility/contractile properties.

The participation of actin in the protein junctional complexes is important as the ratio polymerized/depolymerized actin controls membrane flexibility, which increases when actin polymerization is inhibited. The tight control over this ratio is exercised by four minor proteins: tropomodulin, tropomyosin, αβ-adducin heterodimers [Joshi *et al*., 1991; Katagiri *et al.*, 1996] and dematin (protein 4.9). Tropomodulin–tropomyosin complexes stabilize the

short RBC actin filaments strengthening the spectrin–actin interactions, by capping its slow growing or pointed end filaments [Fowler *et al*., 1993], while dematin (protein 4.9) bundles actin filaments into cables and adducins are involved in both the capping and bundling of actin filaments [Hughes and Bennet, 1995]. Adducins also play a role in the early assembly of the spectrin–actin complexes forming a spectrin–actin–adducin ternary complex.

1.2.2 Haemoglobin

The main function of red cells is oxygen transport to tissues and that is possible thanks to a metalloprotein, haemoglobin, firstly described from Perutz in 1960 [Perutz, 1960]. A mature red blood cell contain around 270 million Hb molecules, which comprise over the 95 % of cytoplasmic proteins. Hemoglobin has an oxygen binding capacity of 1.34 ml O_2 per gram [Dominguez *et al*., 1981], which increases the total blood oxygen capacity seventy-fold compared to dissolved oxygen in blood. In humans this tetrameric and globular protein is formed by one pair of α-like globin chains (α and ζ) and one pair of β-like globin chains (β, ε, γ and δ) stabilized from non covalent bounds. This two different peptide chains are encoded by different genes; in particular, the α-like by a cluster of genes on the short arm of chromosome 16, while the β-like by a cluster of genes on the short arm of chromosome 11. Each globin chain contains an hydrophobic pocket bound to a prostetic heme group, made up of an iron atom at the center of a porphyrin ring. The iron forms four bonds with the porphyrin nitrogens, one bond with an hystidine residue of the polypeptide chain and a sixth bond with exogenous ligands, such as oxygen and carbon monoxide [Bossi and Giardina, 1996]. The heme iron can assume the ferric (Fe^{3+}) or the ferrous (Fe^{2+}) oxidation state and only this latter is able to reversibly bind oxygen [Bossi and Giardina, 1996]. The location of the heme within an hydrophobic pocket allows non covalent bound to occur, ensuring protection of the iron from its autooxidation. The high rate of conservation of this "pocket" in various animal haemoglobins underlies the importance of this region for the stability and function of the molecule itself.

The haemoglobin molecule shows two different conformational states depending on oxygen binding: the deoxygenated one (T state) with low affinity for O_2, and the oxygenated structure (R state) with high affinity for O_2. Inside the erythrocyte, one form would be predominant on the other depending on the localization of the cell in the organism, according to blood circulation in the organism. Indeed, T and R state serve the function of oxygenation of the

tissues thanks to their reciprocal switch. In the last century there were discovered many affectors involved in haemoglobin physiology. In 1903, Christian Bohr firstly demonstrated that protons and carbon dioxide are able to reduce the haemoglobin affinity for O_2 in an heterotropic manner (Bohr effect). This effect has a physiological role in facilitating the oxygen transport in organism from the lungs, in which it binds to haemoglobin, to the tissues, where the gas is finally released. Carbon dioxide that increases during metabolic activities of cells forms carbonic acid and through a balance finally produces protons and bicarbonate. This slow reaction is catalyzed by the enzyme carbonic anhydrase and so the pH rapidly decreases, promoting the dissociation of oxygen from haemoglobin and thus supplying tissues with oxygen. This effect allows tissues to adapt to supply oxygen when it is needed most, expecially in muscular tissues under strong activity. The CO_2 that respiration and lactic fermentation generate, lead blood pH to decrease to around 7.2, leading haemoglobin to release around 10 % more oxygen.

On the contrary, in the lungs, where the partial oxygen pressure (pO_2) is very high (around 100 mmHg), on the contrary, the bound of the gas with haemoglobin produces protons which, combining with bicarbonate, shift the equilibrium to the CO_2 and thus to the exhalation of carbon dioxide through airways.

Later on, in 1976, Reinhold and Ruth Benesch discovered another haemoglobin effector, the 2,3-diphosphoglycerate (2,3-DPG). It is an organophosphate generated in red blood cells by glycolitic pathway and it competes with O_2 for deoxyhemoglobin binding. The action of 2,3-DPG consists in stabilizing the low oxygen affinity state of haemoglobin and so making easier for the protein to release oxygen. This function is particularly important in tissues, that in the circulatory apparatus are the ones that more than all need oxygen and is also potentiated by the Bohr effect. Moreover, particular or pathological conditions such as high altitude, airway obstruction or congestive heart failure tend to cause an accumulation of 2,3-DPG.

However, hemoglobin is responsible not only of O_2 carrying, but of other gases as well. 10% of body content of carbon dioxide is indeed transported by carbaminohemoglobin, where the gas is bounded to globin chains. Nitric oxide is also carried by this protein, by a covalent bound with a thiol group and is released together with O_2.

1.2.3 Erythrocyte metabolism

Red cells lack intracellular organelles and so they are uncapable of protein and lipid synthesis and of oxidative phosphorylation [Bossi and Giardina, 1996]. The main processes for which

energy is needed are the maintenance of osmotic balance through ATP-dependent pumps, enzymes and membrane protection from oxidative stress, and maintenance of Hb iron in ferrous state. Since the main function of erythrocytes is molecular oxygen transport, they are subjected to strong oxidative stress and many functional and structural proteins have to be preserved in reduced state in order to allow the cell survival.

To sustain these functions, erythrocytes rely on two metabolic pathways: Embden-Meyerhoff (glycolysis) and Pentose phosphate pathways. In the case of glycolysis, glucose can be only partially oxidized, producing 2 ATP, 2 NADH and 2 piruvate molecules (Fig. 4).

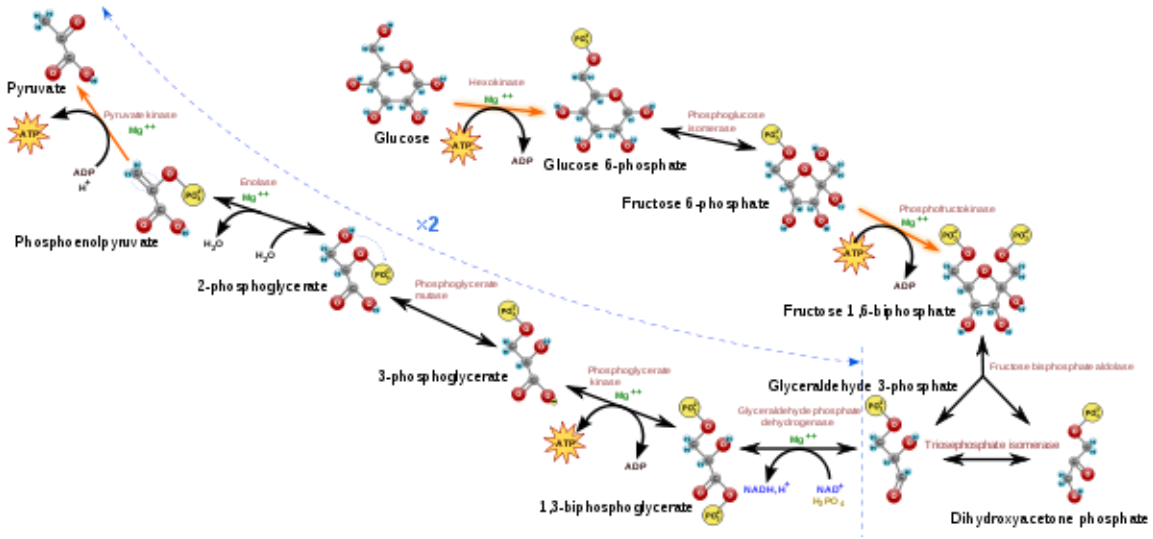

Figure 4. Glycolysis.

ATP is the main energy source for erythrocytes and many enzymes are ATP dependent, such as the Na^+-K^+ pump, a very important enzyme for red cells. It regulates the osmosis equilibrium by extruding Na^+ ions together with water molecules and so, in absence of ATP, the cell swells, becoming not adapted to microcirculation.

NADH that is produced during glycolysis is instead very important for red cells to maintain their physiological role of oxygen transporters. In fact, it acts as electron donor-cofactor for the methaemoglobin reductase enzyme, which is responsible of methaemoglobin conversion to native ferrous haemoglobin form.

The net gain of glycolysis is actually modified in red blood cells from the Rapoport-Luebering pathway that generates 2,3-DPG that is necessary for haemoglobin physiology as previously reported.

Another important metabolic way in red blood cells is the pentose phosphate pathway (PPP) or hexose monophosphate shunt. Around 5-10% of glucose is converted in ribulose-5-phosphate by this way, producing reducing power in the form of 2 NADPH molecules. NADPH is necessary in red blood cells as H^+ donor in the glutathione cycle. This cycle is necessary to continuously reconvert oxidized glutathione (GSSG) in its reduced form (GSH). GSH acts as cofactor of glutathione peroxidase, the enzyme responsible of H_2O_2 scavenging with the production of H_2O molecules (see paragraph 1.5 for a detailed description)**.**

1.3 Erythrocyte storage

1.3.1 Historic evolution

Blood has been stored for human transfusion for more than 90 years and for almost as long the red cells as well [Hess and Schmidt, 2000]. At the beginning of transfusion history, donor and recipient used to be side by side [Hess, 2006] and everything has to happen before clotting intervened. The possibility to store blood as we nowadays know began in 1913 when Lee and Vincent demonstrate that citrate could prevent the coagulation. Since that moment, the donor and the recipient could be separated in space. Following researches have been focused in the look for the optimal amount of citrate without causing any side effect. In 1916, Rous and Turner firstly described the four weeks storage of rabbit red cells in citrate and glucose with minimal haemolysis. The support of glucose to the erythrocyte metabolism allow the donor and the recipient to be separated in time too. The Rous and Turner solution was subsequently used in the World War I. Anyway, prior of the discovery that glucose solution could be autoclaved without caramelization if the pH is 5.8, the only use of citrate was preferred from US Army Medical Corps to avoid bacterial contamination. Moreover, still in the view of military logistic, the introduction of plastic bags in 1950's represented a great advantage because of their lighter weight and resistance to breakage. When the diethylhexyl phthalate (DHEP) was coupled with PVC classic bags, it has been shown a fourfold reduction of haemolysis and the time of storage increased twice. Nowadays it is known that DHEP enters the RBC membrane where it limits membrane loss by microvesiculation. Starting from here, blood banking evolutes in the next years and blood transfusion became soon a planned healthcare activity. At the same time, scientists were working, and still they are, on the improvement of storage time through the use of other molecules in addition to glucose and

citrate, e.g. phosphate to offset its dilutional loss from cells. In 1962, Nakao and colleagues [Nakao *et al*., 1962] described the relationship between loss of RBC viability and their loss of ATP. In fact, addition of adenine and inosine could help storage by cell shape, ATP and viability restoration, extending the storage time to 5 weeks (citrate, phosphate, dextrose, adenine: CPDA-1).

Additive solutions were developed to provide additional volume and nutrients for longer storage and better flow of packed RBCs. They represent the last step in the blood collection and storage system. In 1978, when the Saline Adenine Glucose & Mannitol (SAG-M) additive solution was introduced, the storage period was brought to 42 days, the period still in effect today. Regulatory standards require red blood cells to be stored at 1–6 °C, to keep them from freezing at -0.5 °C which ruptures their membranes, or from getting so warm that they consume all of their nutrients before their expected shelf-life [Hess, 2010]. Stored at room temperature, red cell concentrates become significantly acidotic in about 3 days, but glycolysis and proton accumulation in stored red cells occur about twenty times more slowly at 3° C than at 37 °C. They can also be shipped under wet ice at temperatures up to 10 °C.

Nowadays, whole blood is not used since the use of its single components as plasma, red blood cells (RBCs) concentrates, and platelets (PLTs) in preferred, in order to transfuse patients only with the needed blood fraction. Moreover it has been demonstrated that white blood cells collected with whole blood break down in the cold and their release of proteases and lipases causes damage to the RBC during storage. Therefore, The practice of leucoreduction, by buffy coat removal or leucofiltration, became a fundamental strategy to increase RBC recovery and reduce haemolysis.

1.3.2 Blood fractionation

The most commonly used way to produce haemocomponents is by means of centrifuges (cooled or not, depending on the product). Red blood cell concentrates, in particular, are definitely the most widely transfused blood component and they are used in order to suddenly increase oxygen supply to the tissues. They are obtained starting from whole blood through a centrifugation that allows to separate leuko-platelet layer (buffy coat) from erythrocytes and plasma (Figure 5).

Figure 5. Blood component sedimentation.

After this step, using a cellular separator with optical sensors, the various blood components pass to satellite bags where they can be stored separately.
So, the "mother" bag, containing 450 cc of whole blood and CPD (citrate-phosphate-dextrose) solution is connected with a first satellite bag, designed for red cell collection with CPD and SAG-M, and to a second one, for the plasma. This procedure is called "top and bottom" collection.
Buffy coat remains, instead, in the mother bag. This strategy allows the red cell concentrates to be immediately ready for the use, or to be stored for up to 42 days since the bag contains the SAG-M as well.

1.4 Erythrocyte storage lesions

Storage lesions are the whole changes that occur to blood components during conventional blood bank storage. During the 42 days of storage at 4°C, in fact, red blood cells undergo a series of modifications that can negatively affect transfusional efficiency.
These modifications can be divided into different categories: metabolic, enzymatic, oxidative and physiologic lesions.

1.4.1 Metabolic storage lesions

Metabolism, as previously reported, is centered on glycolysis, the only red cell source of energy. As a consequence, the attempts to increase the duration of their storage are focused on

optimizing their glucose metabolism. Protons produced by glycolysis increase the acidity of red cells and storage solutions as well. Acidosis inhibits in turn the glycolysis because of low pH adverse effect on activity of hexose kinase (HK) and phosphofructokinase (PFK), and so, as glycolysis proceeds, fewer ATP and NADH are produced. Moreover, the inhibition of HK leads to a poor production of NADPH and glutathione, since it is decreased the production of glucose phosphate as well. In this view, the pH of the initial storage solution is also an important determinant of stored red cell metabolism and survival, but it is only generally controlled in storage systems and varies over a modest but clinically significant range [Hess, 2002]. Venous whole blood from the typical healthy donor will have a pH of about 7.35. When collected into CPD (pH 5.5–5.8), the resulting red cell suspension has a pH of about 7.0, but the exact pH can be 0.2 pH units more or less depending on the haemoglobin concentration and volume of the donor blood. However, at any storage pH less than 7.2, the breakdown of 2,3-DPG is favored, and this in turn leads to an initial burst of ATP production.

As a result of these activities, red cell ATP concentrations initially rise as 2,3-DPG is broken down, stay above their initial concentrations for 2–3 weeks, and then decline steadily. The pH typically declines from 7.0 to 6.5, and the rate of glucose consumption decreases by more than 50%. As was mentioned before, the slowing of glycolysis also leads to reduced production of NADH leading to reduced activity of methaemoglobin reductase, and thus the methaemoglobin concentration of stored red cells increases over 6 weeks of storage, typically from about 1–2%. Flux through the hexose-monophosphate shunt also slows, and the concentration of reduced glutathione decreases by up to 80% [Dumaswala *et al*., 2000; Dumaswala *et al*., 2001].

Low temperature profoundly reduces the activity of the major membrane sodium-for-potassium pump, which is ATP-dependent. As a result, the potassium that slowly leaks out of RBCs is not returned, and the concentration of extracellular potassium in the suspending fluid in the red cell bag typically rises at a rate of about 1 mEq/L/day [Hess, 2010].

If units with high concentrations of extracellular potassium are given at high flow rates into central lines or used to prime cardiac bypass machines, they can expose the heart to high concentrations of potassium and cause arrhythmias and sudden death [Hall *et al.*, 1993].

1.4.2 Enzymatic storage lesions

Enzymatic damages are related to the presence of white blood cell (WBC) during storage period. In fact, it has been demonstrated that WBC 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 white blood cells by filtration, so called leukoreduction, can reduce enzymatic damage, thereby improving recovery and reducing 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.

1.4.3 Physical storage lesions

Physical changes mainly involve the RBC shape, through the reorganization of membrane structure or its loss, with production of vescicles and microvescicles. Membrane loss is an ordinary process in RBC maturation as a 140 μm^3 reticulocyte becomes a 90 μm^3 mature erythrocyte [Greenwalt, 2006] that anyway has 50% more membrane than it is needed to contain its volume and the extra membrane is necessary to erythrocyte deformation and squeezing inside the circulatory system. Erythrocytes lose their deformability, or the possibility to temporarily change their shape, as a consequence of a huge reduction of their surphace area to volume ratio. During the storage period, the erythrocyte evolves from

biconcave disc to spiculate shape (echinocytes) and is finally called sphero-echinocytes (Fig. 6).

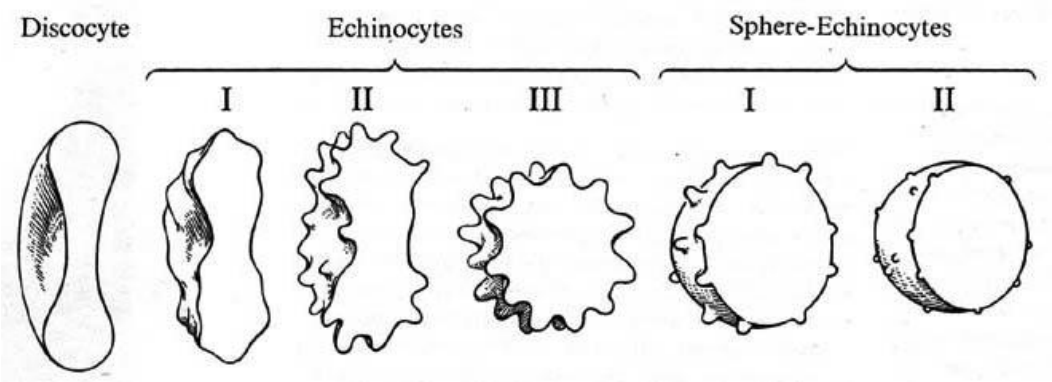

Figure 6. Evolution of a red blood cell during storage.

It has been demonstrated that this process is associated with increased protons, ATP and intracellular calcium concentration. The return toward the normal red cell shape occurs in parallel with increasing ATP concentration, regeneration of 2,3-DPG and the restoration of normal sodium, potassium and calcium gradients. Beyond the spheroechinocyte stage, the cell lose its membrane as microvesicles bud from the tips of spines and this process is not reversible, since the red cell has no mechanism to replace lost membrane. At the end of the storage, the densest cells have lost all extra membrane and have become rigid spheres. The different-sized vesicle populations isolated from the supernatant of stored red cells seem to have different properties. Some are rich in oxidized lipids, most have exposed phosphatidyl serine that normally resides on the inner surface of the membrane, and almost all are deficient in membrane proteins that are attached to the cytoskeleton [Antonelou *et al*., 2009]. The exposed negatively-charged lipids on such vesicles can make them proinflammatory and prothrombotic. Normally, red cell-derived vesicles with exposed phosphotidyl serine are cleared by macrophages as soon as they are made.

1.4.4. Oxidative storage lesions

In erythrocyte aging and lysis process (both *in vivo* and *in vitro*), oxidative stress plays a fundamental role, since the red cell is continuously exposed to oxygen and also rich in polyunsaturated lipids, target of free radicals, and iron, a powerful catalyzer of free radical, through Fenton reaction (Fig. 7).

$$Fe^{3+} + O_2^{\cdot -} \longrightarrow Fe^{2+} + O_2$$
$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\cdot} + OH^-$$

Figure 7. Fenton reaction.

Usually the cell is able to protect from free radicals thanks to the presence of scavenger substances and enzymatic systems (see next paragraph for a detailed description). *In vitro*, anyway, the erythrocyte is unable to regenerate antioxidant defences and so the production of free radical increase inexorably with time. At the end, this entails huge variation at the level of permeability, antigenicity and rheological properties. The main target of oxidative injury is haemoglobin that, once is oxidized, binds irreversibly to some membrane proteins bringing to alterations in the normal organization of cytoskeleton. Nevertheless, haemoglobin itself is a source of oxidative active molecules. In figure 8 are summarized the major haemoglobin oxidative pathways and their link to membrane damage.

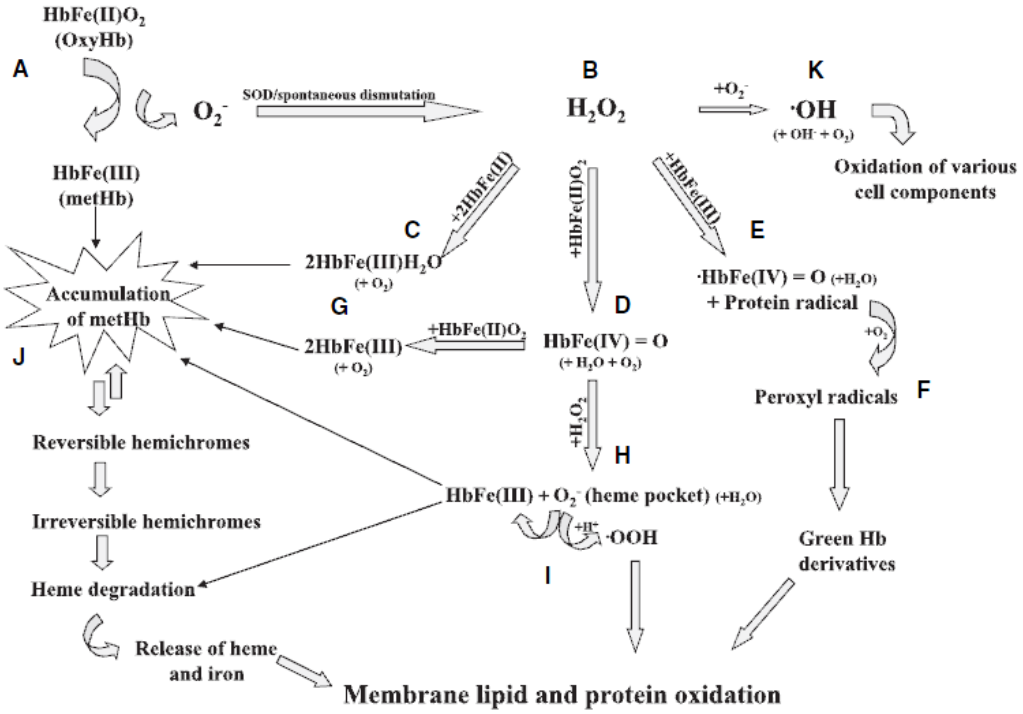

Figure 8. The major haemoglobin oxidative pathways and their link to membrane damage (from Kanias T. and PJ Acker, 2010).

Anytime haemoglobin binds an atom of oxygen, there is a migration of charge from the heme iron to the oxygen such that the structure of oxyHb is $Fe^{3+}\ O_2^-$ [Kanias and Acker, 2010]. Oxy

Hb is a fairly stable molecule that slowly auto-oxidizes to met-Hb and involves the dissociation of the oxygen without electron transfer to yield superoxide O_2^- and met-haemoglobin [HbFe(III)]. Superoxide anion is a reactive oxygen species (ROS) which is toxic if it is not eliminated. Methaemoglobin is a fairly stable molecule and easily can release the heme from the globin heme pocket and then iron from the heme itself through intermediates called hemicromes. As mentioned above, iron is one of the intermediate in Fenton reaction and its presence, together with superoxide anion, leads to the production of hydroxyl radical. Superoxide anion, by spontaneous dismutation or mediated by superoxide dismutase (SOD) enzyme, is converted to hydrogen peroxide that in turn can lead to accumulation of metHb or to the production of other kinds of oxygen radicals such as hydroxyl, perhydroxyl and peroxyl (·OH, ·OOH and ROO·, respectively). In normal conditions, these phenomena are totally reversible, because metHb is continuously reduced through NADH coming from glycolysis and NADH methaemoglobin reductase; at the same time, thiol groups are regenerated thanks to GSH produced by NADPH dependent-glutathione reductase. ·OH is the major responsible for lipid peroxidation (lipoperoxidation). The attack of the radical to the lipid molecule produces a lipid radical that is unstable and reacts in turn with O_2, leading to the production of peroxyl-fatty acid radical. This is as well an unstable species that reacts with another free fatty acid, producing a different fatty acid radical and a lipid peroxide, or a cyclic peroxide if it had reacted with itself. This cycle continues, as the new fatty acid radical reacts in the same way. Some of the first oxidation products can easily react to secondary oxidation products like malondialdeyhde (MDA), 4-hydroxy-2-,3-trans-nonenal (HNE), isoprostanes or oxysterols. These secondary oxidation products can lead to further damage by cross linking proteins. Lipids, moreover, can be oxidative dealkylated becoming active diakyl-glycerols such as PAF [Silliman *et al*.,1998].

Proteins are one of the major macromolecule category affected by age-related damage, since ROS can lead to oxidation of amino acid residue side chains, formation of protein-protein cross-linkages, and oxidation of the protein backbone resulting in protein fragmentation. It is estimated that almost every third of protein in a cell is dysfunctional as enzyme or structural protein due to oxidative damage [Poon *et al*., 2004].

The attack of ROS against proteins modifies lysine, arginine, proline and histidine amino acid residues generating carbonyl moieties, which have been identified as an early marker for protein oxidation and are used as a measure of protein damage [Levine *et al*., 1980].

In addition, carbonyl groups may be introduced into proteins by reactions with aldehydes (4-hydroxy-2-nonenal, malondialdehyde) produced during lipid peroxidation [Uchida and Stadman, 1993] or with reactive carbonyl derivatives (ketoamines, ketoaldehydes, deoxyosones) generated as a consequence of the reaction of reducing sugars or their oxidation products with lysine residues of proteins [Kristal and Yu, 1992]. An age related increase in the protein carbonyl concentration was reported for tissues like heart, muscle or brain [Floyd *et al.*, 2002; Stadtman 2001], as well as in plasma of healthy people. Although, proteolytic systems exist to remove oxidized proteins following severe oxidative stress, the decrease in the proteolytic degradation and accumulation of mis-folded proteins may be the cause and/or the consequence of many disorders and aging.

1.5 Antioxidative enzymatic defense

Several enzymes have evolved in aerobic cells to overcome the damaging effects of ROS. They are significantly used to maintain the redox balance during oxidative stress and are collectively called as endogenous antioxidative enzymes. Superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT) and peroxiredoxins (Prxs) are the main endogenous enzymatic defense systems of all aerobic cells. They give protection by directly scavenging superoxide radicals and hydrogen peroxide, converting them to less reactive species.

SOD catalyzes the dismutation of superoxide radical ($\cdot O_2$) to hydrogen peroxide (H_2O_2). Although H_2O_2 is not a radical, it is rapidly converted by Fenton reaction into ·OH radical which is very reactive. Among various antioxidant mechanisms in the body, SOD is thought to be one of the major enzymes that protect cells from ROS.

Glutathione peroxidase (GPx) neutralizes hydrogen peroxide by taking hydrogens from two GSH molecules, resulting in two H_2O and one GSSG. The enzyme glutathione reductase then regenerates GSH from GSSG with NADPH as a source of hydrogen (Fig. 9).

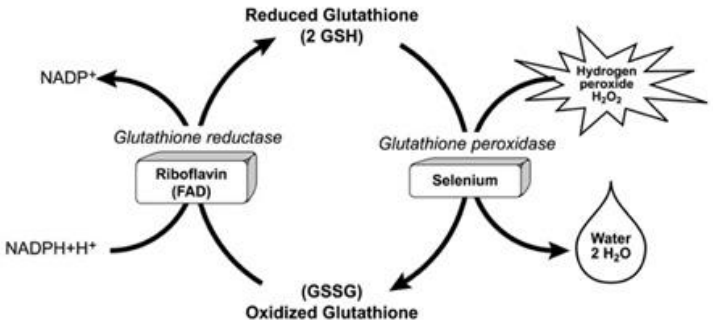

Figure 9. Glutathione cycle.

The deficient production of NADPH, that is one of the metabolic aspects of storage, and as a consequence, of reduced glutathione, bring to a decrease in resistance to oxidative stress, affecting the correct physiology of the erythrocyte.

Another important part of the enzymatic defense system is catalase (CAT). CAT is one of the most active catalysts produced by nature and it is a tetrameric heme-containing enzyme found in all aerobic organisms. Because of its wide distribution, evolutionary conservation and capacity to rapidly degrade hydrogen peroxide, it has been proposed that CAT plays an important role in systems which have evolved to allow organisms to live in aerobic environments [Scandalios, 2005]. A very interesting mechanism shown by the CAT depending upon the concentration of H_2O_2 is its dual function. CAT neutralizes the H_2O_2 in a concentration dependent manner. At low concentrations (<1 μM) of H_2O_2, it acts in this manner:

$RH_2 + H_2O_2 \rightarrow R + 2H_2O$,

while at higher concentrations of substrate, CAT decomposes toxic hydrogen peroxide at an extremely rapid rate using the "catalatic" reaction in which H_2O_2 acts as both an acceptor and donor of hydrogen molecules:

$2H_2O_2 \rightarrow 2H_2O + O_2$.

Another property that makes CAT unique among H_2O_2 degrading enzymes is that it degrades H_2O_2 without consuming cellular reducing equivalents. Hence, CAT provides the cell with a very energy efficient mechanism to remove hydrogen peroxide. Therefore, when cells are stressed for energy and are rapidly generating H_2O_2 through "emergency" catabolic processes, H_2O_2 is degraded by CAT in an energy-efficient manner.

Therefore activity of CAT is one of the important biomarker of oxidative stress.
Peroxiredoxins are another important class of active enzymes against oxidative stress and in the next paragraph there is a deeper coverage about them.

1.6 Peroxiredoxin

1.6.1. Mechanism of regulation of Peroxiredoxins

Peroxiredoxins (Prxs) [Chae *et al*., 1994; Chae *et al*., 1994] are an ubiquitous protein family with antioxidant properties and, more precisely their scavenger activity is thiol specific. Prxs exert their role in the cell through their peroxidatic activity ($ROOH + 2e^- \rightarrow ROH + H_2O$) so that hydrogen peroxide, peroxynitrite and a wide range of hydroperoxides (ROOH) are reduced and so detoxified [Hofmann *et al*., 2002; Jacobson *et al*., 1989; Poole and Ellis, 1996; Bryk *et al*., 2000; Peshenko and Shichi, 2001]. This class of enzymes has been identified in yeast, plants and animals, including protozoa and parasitic helminthes and most of the Eubacteria and Archea [Wood *et al*., 2003]. In spite of being localized mostly in the cytosol, Prxs are found also in mitochondria, chloroplasts and peroxisomes, associated with nuclei or membranes and, in at least one case, exported [Hofmann *et al*., 2002; Jacobson *et al*., 1989; Poole and Ellis, 1996; Bryk *et al*., 2000; Peshenko and Shichi, 2001; Jin and Jeang, 2000]. Prxs are produced at high levels in the cells, in fact are among the ten most abundant proteins in *Escherichia coli* [Link, 1997], second or third most abundant protein in the erythrocytes and compose the 0,1-0,8 % of soluble proteins in other mammalian cells [Moore, 1991]. Most of the organisms have more than one isoform of Prx and at least six Prxs have been identified in mammals (Table 1).

Prx subtype	PrxI (2-Cys)	PrxII (2-Cys)	PrxIII (2-Cys)	PrxIV (2-Cys)	PrxV (atypical 2-Cys)	PrxVI (1-Cys)
Previous nomenclature	TPx-A NKEF A MSP23 OSF-3 HBP23 PAG	TPx-B NKEF B PRP Calpromotin Torin Band-8 TSA	AOP-1 SP22 MER5	AOE372 TRANK	AOEB166 PMP20 AOPP	ORF06 LTW4 AOP2
Polypeptide length	199 aa	198 aa	256 aa (cleaved at 63–64)[a]	271 aa (cleaved at 36–37)[a]	214 aa (cleaved at 52–53)[a]	224 aa
Human chromosomal location	1q34.1	13q12	10q25–q26	10p22.13	11q13	1q23.3
Cellular location	Cytosol, nucleus	Cytosol, membrane	Mitochondria	Cytosol, Golgi, secreted	Mitochondria, peroxisome, cytosol	Cytosol
Genbank SwissProt	AAA50464 tdx2_human P35703	AAA50465 tdx1_human P32119	BAA08389 tdxm_human P30048	AAB95175 tdxn_human Q13162	AAF03750 aopp_human P30044	BAA03496 aop2_human P30041
Interactions with proteins and other ligands	c-Abl Presenilin-1 Heme Macrophage migration inhibitory factor Cyclophilin	Protein 7.2b (stomatin) Presenilin-1 Erythrocyte membrane Cyclophilin	Cyclophilin Abrin A-chain	Heparin Cyclophilin	DNA Cyclophilin	Cyclophilin

Abbreviation: aa, amino acids.
[a]These proteins are post-translationally processed.

Table 1. Six subclasses of Peroxiredoxins (Prxs) from mammals.

As previously mentioned, peroxidatic functions of Prxs are partially overlapped to the one of GPx and CAT, although it has been suggested that their moderate catalytic efficiencies compared with those of the other two enzymes, make their importance as peroxidases questionable. Anyway, the high abundance of Prxs in many cellular type and in such a wide range of cells, allowed to appreciate the Prxs as one of the most important detoxifying enzyme class in the cell. All Prxs to date conserve an active-site Arg, which would lower the pKa of the peroxidatic cysteine somewhat by stabilizing its thiolate form.

Prxs use redox-active cysteines to reduce peroxides and were originally divided into two categories, the 1-Cys and 2-Cys Prxs, based on the number of cysteinyl residues directly involved in catalysis [Chae, 1994] (Fig 10). The last class of Prxs, the 1-Cys Prxs, conserves only the peroxidatic cysteine and does not contain a resolving cysteine [Choi, 1998]. Their cysteine sulfenic acid generated on reaction with peroxides is presumably reduced by a thiol-containing electron donor, but the identity of this redox partner is not clear yet (although proposed electron donors have included glutathione, lipoic acid and cyclophilin [Hofman *et al.*, 2002, Peshenko and Shichi, 2001]. By analogy, one donor thiol probably forms a transient mixed disulfide bond with the enzyme, followed by its reduction by a second donor thiol, thus recycling the enzyme.

Structural and mechanistic data now support the further division of the 2-Cys Prxs into two classes called the ‘typical’ and ‘atypical’ 2-Cys Prxs.

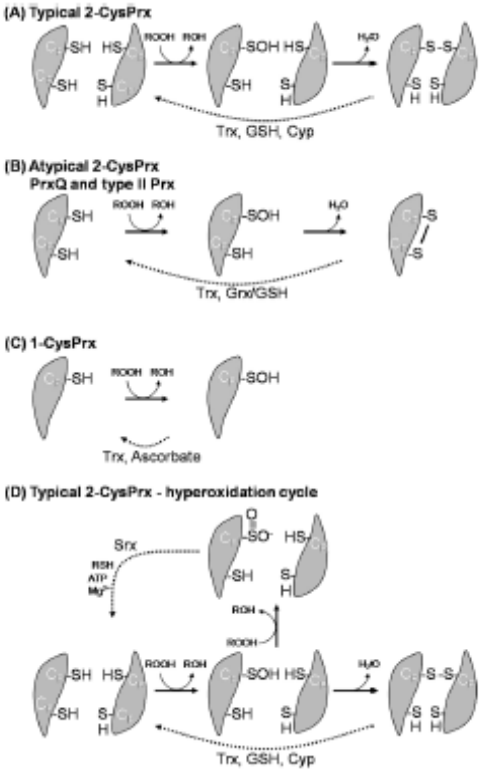

Figure 10. Catalytic mechanisms of the three classes of Prxs. The peroxidatic cysteine (CP) initially reacts with peroxide substrate. In the typical 2-CysPrxs, CP reacts with resolving Cys (CR) on the second dimer subunit of the dimer (A), while in atypical 2-CysPrxs, resolving Cys is localized on the same polipeptide chain (B). The Cys-SOH of the 1-CysPrx is directly regenerated through an electron donor in thiol form (C).
Cyp: cyclophyilin; Grx: glutaredoxin; GSH: Glutathione; ROOH: peroxide; RSH: electron donor; Srx: sulfiredoxin; Trx: thioredoxin.

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Both classes share the conservation of two redox active sites in the protein, the peroxidatic cysteine (generally near residue 50) and the resolving cysteine (near residue 170) [Hofmann *et al*., 2002].

In the case of atypical 2-Cys Prxs, the detoxification of peroxide entail the formation of a intramolecular disulphide bond among the two cystein residues of the same monomer. To recycle the disulfide, known atypical 2-Cys Prxs appear to use thioredoxin as an electron donor [Seo *et al*., 2000] and reducing equivalents derived from NADPH.

The typical 2-Cys Prxs are instead obligate homodimers [Hirotsu *et al*., 1999; Schröder *et al*., 2000; Alphey *et al*., 2000; Wood *et al*., 2002] because the second step of the peroxidase reaction involves the peroxidatic cysteine sulfenic acid (Cys–SROH) from one subunit and the resolving cysteine (Cys–SRH) located in the C-terminus of the other subunit (Fig. 10). This condensation reaction results in the formation of a stable intersubunit disulfide bond,

which is then reduced by one of several cell-specific disulfide oxidoreductases (e.g. thioredoxin, AhpF, tryparedoxin or AhpD [Poole *et al*., 2000], completing the catalytic cycle. Although the resolving cysteines of typical and atypical 2-Cys Prxs are not conserved in sequence, they are functionally equivalent.

1.6.2. Oligomerization of Peroxiredoxins

The first reference or Prx oligomerization came through transmission electron microscopy (TEM), that could reveal complexes with tenfold symmetry of an abundant protein isolated from erythrocytes [Harris, 1969] (Fig. 11a).

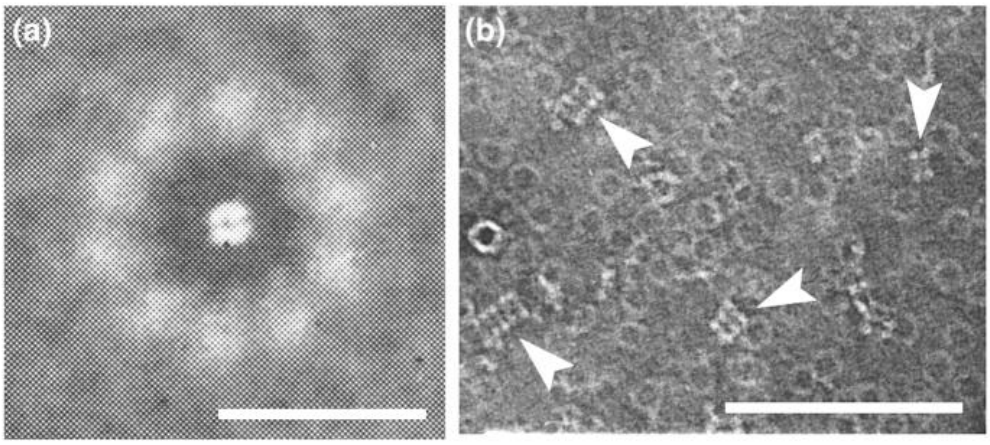

Figure 11. Transmission electron microscopy studies of Peroxiredoxin (Prx II).

This protein was further identified as a typical 2-Cys Prx [Harris *et al*., 2001]. In the TEM reports, it was observed that under certain conditions, PrxII and the related PrxIII could also form higher-order multimers by stacking into columns of various lengths (Fig. 11b).
In 2000 Schröder and colleagues demonstrated that the decamer was actually formed by a doughnut arrange of five dimers [Schröder *et al.*, 2000] (Fig. 12).

Figure 12. Quaternary structure of decameric state of PrxII in human red blood cell. The lines separate the 5 dimeric units.

The oligomeric properties of several typical 2-Cys Prxs in solution have been studied using gel filtration, light scattering and analytical ultracentrifugation. Factors shown to promote oligomerization in typical 2-Cys Prxs include high or low ionic strength [Kitano *et al*., 1999], low pH [Kristensen *et al*., 1999], high magnesium [Kato *et al*., 1985] or calcium [Allen *et al*., 1979, Plishker *et al*., 1992] concentrations, reduction of the redox-active disulfide center [Schröder *et al*., 2000], and 'overoxidation' of the peroxidatic cysteine to a sulfinic acid (Cys–SO_2H) [Schröder *et al*., 2000]. Reduction of the active-site disulfide of typical 2-Cys Prxs is emerging as the primary factor in the stabilization of the decameric forms of these enzymes; a direct link between redox state and oligomerization state was recently established through analytical ultracentrifugation of several bacterial 2-Cys Prxs [Wood et al., 2002; Reynolds *et al.*, 2002] and human PrxII [Schröder *et al*., 2000], as well as earlier gel-filtration studies of porcine PrxII [Schröder *et al*., 1998]. The dynamic equilibrium between oligomer assembly and disassembly has been extensively studied, however it is still intriguing, especially because the adopted different conformation states are linked to switches in function. An important feature of mammalian 2-Cys is that high levels of peroxide produce their inactivation through the overoxidation of the peroxidatic cysteine to sulfinic (Cys-SpO_2H) or sulfonic (Cys-SpO_3H) acid [Hall *et al*., 2009; Yang *et al*., 2002]. Although the sulfinic acid form was originally thought to be biologically irreversible, sulfiredoxins (Srxs) and possibly sestrins are able to revert Cys-SpO_2H to the reduced state in an ATP-dependent

reaction [Biteau *et al*., 2003; Jönsson and Lowter, 2007]. The existence of such a resurrection activity supports a physiological role for the overoxidized form of Prxs and, more precisely, it has been suggested that overoxidation allows intracellular accumulation of H_2O_2 which can then function as a signal transducer for various pathways [Biteau *et al*., 2003; Kang *et al*., 2005]. On the other hand, as mentioned before, the peroxidatic cysteine is also the molecular switch responsible for the redox-sensitive oligomerization of 2-Cys Prxs. In fact, within the dynamic cycle, the decamer breaks down releasing free disulfide-bonded dimers, whereas hyperoxidation seems to freeze the Prx protein as a decamer or might even promote further aggregation with abolishment of peroxidase activity and exhibition of chaperone function [Barranco-Medina *et al*., 2009; Moon *et al*., 2005; Meissner *et al*., 2007]. At any rate, despite the numerous observations accumulated in various cell lines and organisms, very little is still known in human erythrocytes where the PrxII was shown to be remarkably sensitive to oxidative stress [Low *et al.*, 2007]. Recent investigations performed on erythrocytes brought to light a peculiar behavior of PrxII with respect of that reported for other cells [Low *et al*., 2007], making of extreme interest the understanding of the redox induced changes in oligomeric state of this protein in such a system. Indeed, erythrocytes represent very unique cells where high rates of reactive oxygen species (ROS) are constantly formed due the interaction of heme-containing hemoglobin and oxygen. For this reason they have a broad repertoire of antioxidants to counter the potentially detrimental effect of oxidative insult.

1.7 Aim of the thesis

Red blood cells can be stored, in standard conditions, for a limited span of time, during which they undergo a series of biochemical and physico-chemical changes that could affect the transfusional efficacy. When stored red cells are transfused to a receiver, some of them remain in the bloodstream, while others are removed within few hours. The reason has to be searched in the so called storage lesions. oxidative stress and proteolytic degradation seems to play a key role during the aging process and the consequent erythrocytes lysis. Storage condition avoid the red cell to cope with the increased amount of free radical that, in turn, are able to induce an oxidative stress on lipids and proteins. This process bring to the activation of proteolytic enzyme and to a wider protein fragmentation leading the cytoskeleton to accumulate severe damages to its protein. The morphological modifications lead to huge alterations to permeability and rheological properties of the erythrocyte, causing an early

aging of red cell that impair their usage in transfusional therapy. This PhD thesis would evaluate protein alterations of red blood cell citoskeleton, during the storage period of 42 days. The aim is the identification of potential of oxidative stress and aging condition biomarkers. To this purpose it will be performed a biochemical analysis of oxidative modifications to cytoskeletal proteins at days 0, 14, 28, and 42 storage days (4°C in saline-adenine-glucose-mannitol, SAG-M). In particular, this thesis suggest the Peroxiredoxin protein (PrxII) as cytosolic protein that during storage migrates to the membrane, as a potential biomarker of oxidative condition of stored red blood cell. Ghost membrane suspension would be treated in reducing and non reducing conditions, since the thiolic groups are the main target of oxidations. It will be also evaluated the condition of the ghosts in presence or absence of oxygen (aerobic and anaerobic storage, the latter with helium addition, in order to have an inert atmosphere in which reactive oxygen species cannot be generated. Moreover, with the aim to demonstrate the copartnership of oxidative stress phenomena in the oligomeric conformation of PrxII, it will compared, through native electrophoresis technique coupled with immunoblotting, protein extract obtained from fresh erythrocytes (control, 0 days of storage) with aged erythrocytes (after 28 days of storage). Functional assays will be also performed, in order to evaluate eventual defense roles against oxidative damage.

Chapter 2

Materials and methods

2.1 Sampling

Whole blood (450 mL ± 10%) was collected from nine units of healthy volunteer donors into CPD anticoagulant (63 mL). After separation of plasma and buffy coat by centrifugation, leukoreduced RBCs were suspended in 100 mL of SAGM solution. We studied RBC units collected from 9 donors [male = 5, female = 4, age 48 ± 11.5 (mean ± S.D.)] in the middle region of Italy. RBC units were stored under standard blood bank conditions (4 ± 2°C) and samples were removed aseptically for the analysis after 0 and 28 days of storage. Density-fractionated RBCs were prepared using Percoll (Sigma Aldrich, St. Louis, MO, USA) discontinuous gradients as described by Alderman *et al*. [1980], and two fractions were considered according to red cell density (fraction F1: density ± 1.10; fraction F2: density ± 1.11).

2.2 Deoxygenating treatment

To determine the effects of anaerobic RBC storage, 60-mL aliquots of RBCs were sampled from each RBC unit by centrifugation (600 x *g*, 5 min) and transferred into 150-mL polyvinylchloride bags (PL146, Baxter Healthcare, Round Lake, IL). SAGM (40 mL) additive solution (AS) was added to each of the 150-mL bags, which were stored at 4°C. To obtain oxygen depletion each bag was filled with ultrapure helium through a 0.22-mm sterile filter and put under gentle horizontal agitation for 30 minutes at 4°C; the gas was then expressed out. This process was repeated five times. The deoxygenation of Hb was verified by spectrophotometric analysis. To prevent reoxygenation, deoxygenated bags were stored in a homemade anaerobic chamber filled with 100% ultrapure helium. The chamber was refilled with helium every day during the 42 days of storage. Oxygen-depleted RBCs and standard RBCs were stored for 42 days under standard blood bank conditions (4 ± 2°C). During storage 10-mL samples were collected aseptically from each RBC unit on Days 0, 7, 14, 21, 28, and 42.

2.3 RBC membrane preparation and trapping of PrxII in its native state

Extraction of human erythrocyte membrane and cytosol proteins was performed based on the conventional method as described by Olivieri *et al*. [2001] with some modifications. The erythrocytes were isolated by centrifuging twice at 1000g for 10 min. Packed cells were

washed three times in 5 mM phosphate buffer pH 8.0, containing 0.9% w/v NaCl; then, they were centrifuged at 300 ×g for 10 min, at 4°C. Erythrocytes were resuspended in 1 mL PBS containing 100 mM N-ethylmaleimide (NEM), to avoid possible oxidation artifacts during cell preparation [Low *et al.*, 2007]. After 15 min of incubation at room temperature, cells were pelleted and then lysed with 9 vol of cold 5 mM phosphate buffer pH 8.0 containing 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF) and 100 mM NEM. Cytosol was collected after centrifugation at 17,000×g for 20 min at 4 °C and its protein content was estimated by the DC protein assay method (Bio-Rad, Hercules, CA, USA). Membranes were washed with the same buffer until free of hemoglobin and then, in order to remove non-specifically membrane-bound cytosolic proteins, were washed three times with 0.9% w/v NaCl and collected at 17,000×g, for 20 min at 4°C. Protein content was estimated by the bicinchoninic acid method [Smith *et al*., 1985] and ghosts prepared in this way were used for the following steps.

2.4 Gel electrophoresis

2.4.1. 1D/SDS-PAGE

Electrophoretic analyses of the RBC proteins were carried out on a continuous system of polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS-PAGE) using a 5% to 16% linear acrylamide gradient gel (30 mg protein/lane) according to Laemmli [1970]. To prepare RBC membranes for electrophoresis, membrane suspensions were treated with an equal volume of solubilization buffer (0.125 mol/L Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 0.053% bromophenol blue) containing either 200 mmol/L dithiothreitol when working under reducing conditions or 100 mmol/L NEM in oxidizing conditions. Proteins were visualized by sensitive colloidal Coomassie brilliant blue G-250 stain. Stained gel images were digitized using a high-resolution scanner (ImageScanner II, GE Healthcare, Uppsala, Sweden) with a resolution of 300 dpi and 16-bit grayscale pixel depth. Image analyses were performed using computer software (Quantity One 4.6.3, Bio-Rad, Hercules, CA). For each protein of interest, the relative amount (as a percentage of the total) was quantified by densitometry. All samples were analyzed in three technical replicates. One-dimensional SDS-PAGE protein bands were selected for profile analysis only if they were found and positively assigned in all of the three replicates of each sample. To compensate for subtle differences in sample loading, the volume of each band was normalized to the total volume of bands.

2.4.2. Clear native PAGE of cytosolic proteins

RBC cytosolic protein extracts were loaded onto a 0.75mm thick 5% e 12.5% w/v acrylamide gradient gel. The procedure was carried out according to Schägger *et al*. [1994] with some modifications. 10 ml of sample buffer (0.1 M Bis-Tris-HCl pH 7, 0.5 M 6-aminocaproic acid, 30% w/v sucrose and 0.001% w/v Ponceau red) containing 100 mM NEM were added to 100 µl of cytosol sample. 100 µg of total protein were loaded onto each lane. The molecular weight of the bands was determined thanks to the High Molecular Weight calibration kit for electrophoresis (GE Healthcare, Uppsala, Sweden). The run was carried out at 4 °C and voltage was increased every 30 min by 25 V starting from 75 to 200 V. 200 V were then maintained for 2 h.

2.4.3. Blue native PAGE of membrane proteins

Ghost membrane protein extract was processed according to Schägger and von Jagow [1991] with some modifications. The membranes were pelleted at 18,000×g at 4°C for 10 min and resuspended in 25BTH20G [20% w/v glycerol, 25 mM Bis-Tris-HCl pH 7, and 25% w/v Pefabloc (Sigmae Aldrich, St. Louis, MO, USA)]. An optimized amount of detergent of 1 g n-docecyl-β-D-maltoside (DDM) per gram of protein was used for erythrocyte membrane protein solubilization. The solubilization occurred on ice with continuous vortexing for 3 min. The solubilized proteins were collected after centrifugation at 18,000×g for 10 min at 4°C and their concentration was estimated by the DC protein assay method (Bio-Rad). 100 mg of proteins (in 30 ml of solubilization buffer) were loaded onto a 0.75mmthick 3.5% e 14% w/v acrylamide gradient gel after the addition of 3 ml of sample buffer [0.1M Bis-Tris-HCl pH 7, 0.5 M 6-aminocaproic acid, 30% sucrose and 5% w/v Serva blue G]. N-ethylmaleimide (NEM) at the final concentration of 100 mM was also added.

2.4.4. 2D CN/BN-SDS-PAGE

Strips from the 1D native electrophoresis were cutted and equilibrated for 30 min in agitation in presence of 50 mM Tris-HCl pH 8.8, 4% SDS, 30% glycerol and 6 M urea (non-reducing conditions). Alternatively, two consecutive incubation steps of 15 min each were performed in the equilibration buffer [50 mM Tris-HCl pH 8.8, 4% SDS, 30% glycerol, 6 M urea] with 3% DTT first and 12% iodacetamide after (reducing conditions). For the second dimension, strips were loaded on a 14% acrylamide SDS gel and covered with cathode buffer with 0.5%

agarose. The molecular weight of the proteins was determined by the Wide Range Sigma Marker protein standard (Sigma-Aldrich, St. Louis, MO, USA).

2.5 Immunoblotting

Proteins (30 µg of protein/lane) were resolved by reducing and non-reducing 14% SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride membrane. To reduce the likelihood of false positives, blocking was performed for 2 hours at room temperature in 5% (wt/vol) non-fat dried milk in Tris-buffered saline. Incubation with antibodies anti-human PrxII was performed overnight at 4°C in 1% (wt/vol) bovine serum albumin in Tris-buffered saline/0.1% Tween 20. Bands were detected with goat antirabbit horseradish peroxidase using enhanced chemiluminescence reagents and digitized with a high-resolution scanner (ImageScanner II, GE Healthcare). Quantification of band intensities was performed with analysis software (Quantity One 4.6.3, Bio-Rad), using an internal control of human recombinant PrxII protein. The amount of PrxII of each sample was determined as a ratio between the sample value and the internal control.

2.6 RBC membrane lipoperoxidation

Malondialdehyde (MDA) levels were estimated in RBCs following the Stocks and Dormandy's method with some modifications [Stocks and Dormandy's 1971]. Briefly, 0.2 mL of RBCs was suspended in 3.0 mL of Krebs's Ringer phosphate buffer solution (pH 7.4), and 1 mL of the cell suspension was treated with 1 mL of 10% trichloroacetic acid and centrifuged at 1000 x *g* for 5 minutes. One milliliter of supernatant was then mixed with 1 mL of 0.67% thiobarbituric acid and heated over a water bath for 20 minutes at 85 to 90°C. The solution was cooled and read against a complementary blank at 532 nm (optical density [OD]1) and 600 nm (OD2). A blank was prepared separately without RBCs. The net OD was calculated after subtracting absorbance at OD2 from that at OD1. The MDA level was determined from the standard plot and expressed as nmol/mL RBCs.

2.7 In gel PrxII activity assay

The in-gel assay for the PrxII peroxidase activity was conducted as described by Kang et al. [2006] with some modifications. After the native electrophoresis, the gel strips were

incubated in staining solution (50 mM sodium acetate buffer pH 5.0, 2 mM o-dianisidine dihydrochloride, 1.15mM sodium azide and 0.006% v/v H2O2) in the dark at room temperature until red-brown bands appeared. To evaluate the ability of PrxII to scavenge also organic peroxides, gel assay was performed replacing 0.006% v/v H2O2 with 0.01% v/v t-butyl hydroperoxide in staining solution. Before assaying, gel strips were incubated with Drabkin's reagent (potassium ferricyanide/potassium cyanide) to inhibit the pseudoperoxidase activity of Hb.

2.8 In-gel digestion

Protein bands observed in SDS-PAGE were carefully excised from Coomassie-stained gels and subjected to in-gel trypsin digestion according to Shevchenko and coworkers, with minor modifications [2007]. The gel pieces were swollen in a digestion buffer containing 50 mmol/L NH_4HCO_3 and 12.5 ng/mL trypsin (modified porcine trypsin, sequencing grade, Promega, Madison, WI) in an ice bath. After 30 minutes, the supernatant was removed and discarded, 20 mL of 50 mmol/L NH_4HCO_3 was added to the gel pieces, and digestion was allowed to proceed at 37°C overnight. The supernatant containing tryptic peptides was dried by vacuum centrifugation. Before mass spectrometric analysis, the peptide mixtures were redissolved in 10 mL of 5% formic acid.

2.9 Protein identification by tandem mass spectrometry

Peptide mixtures were separated using a nanoflow high performance liquid chromatography system (Ultimate, Switchos, Famos, LC Packings, Amsterdam, the Netherlands). A sample volume of 10 mL was loaded by the autosampler onto a homemade 2-cm fused silica precolumn (75 µm inside diameter, 375 µm outside diameter; Reprosil C18-AQ, 3 µm, Dr Maisch GmbH, Ammerbuch-Entringen, Germany) at a flow rate of 2 mL/min. Sequential elution of peptides was accomplished using a flow rate of 200 nL/min and a linear gradient from Solution A (2% acetonitrile, 0.1% formic acid) to 50% of Solution B (98% acetonitrile, 0.1% formic acid) in 40 minutes over the precolumn in line with a homemade 10- to 15-cm resolving column (75 µm inside diameter, 375 µm outside diameter; Reprosil C18-AQ, 3 µm, Dr Maisch GmbH). Peptides were eluted directly into a high-capacity ion trap (Model HCTplus, Bruker-Daltonik, Bremen, Germany). Capillary voltage was 1.5 to 2 kV and a dry gas flow rate of 10 L/min was used with a temperature of 230°C. The scan range used was

from 300 to 1800 m/z. Protein identification was achieved by searching the National Center for Biotechnology Information nonredundant database (NCBInr, Version 20090424, http://www.ncbi.nlm.nih.gov) using theMascot program (in-house version 2.2,Matrix Science, London, UK). The following variables were adopted for database searches: complete carbamidomethylation of cysteines and partial oxidation of methionines, peptide mass tolerance ± 1.2 Da, fragment mass tolerance ± 0.9 Da, and missed cleavages 2. For positive identification, the score of the result of (-10 X Log[P]) had to be over the significance threshold level ($p < 0.05$).

2.10 Statistical analysis

Descriptive statistics are presented as arithmetic mean ± standard deviation. Paired student t tests were carried out as indicated in figure legends with p values of less than 0.05 used to reject the null hypothesis.

Chapter 3

Results

3.1 Presence of cytoplasmic proteins in ghost membranes

To study storage-dependent remodeling of RBC membranes, in terms of both changes in the association of cytosolic proteins and aggregation or degradation of membrane proteins,8 we analyzed the ghost membranes of RBCs after 0, 14, 21, 28, and 42 days of storage. All samples were subjected to SDS-polyacrylamide gradient gels under reducing and non-reducing conditions (Fig. 13A) followed by mass spectrometry analysis for protein identification.

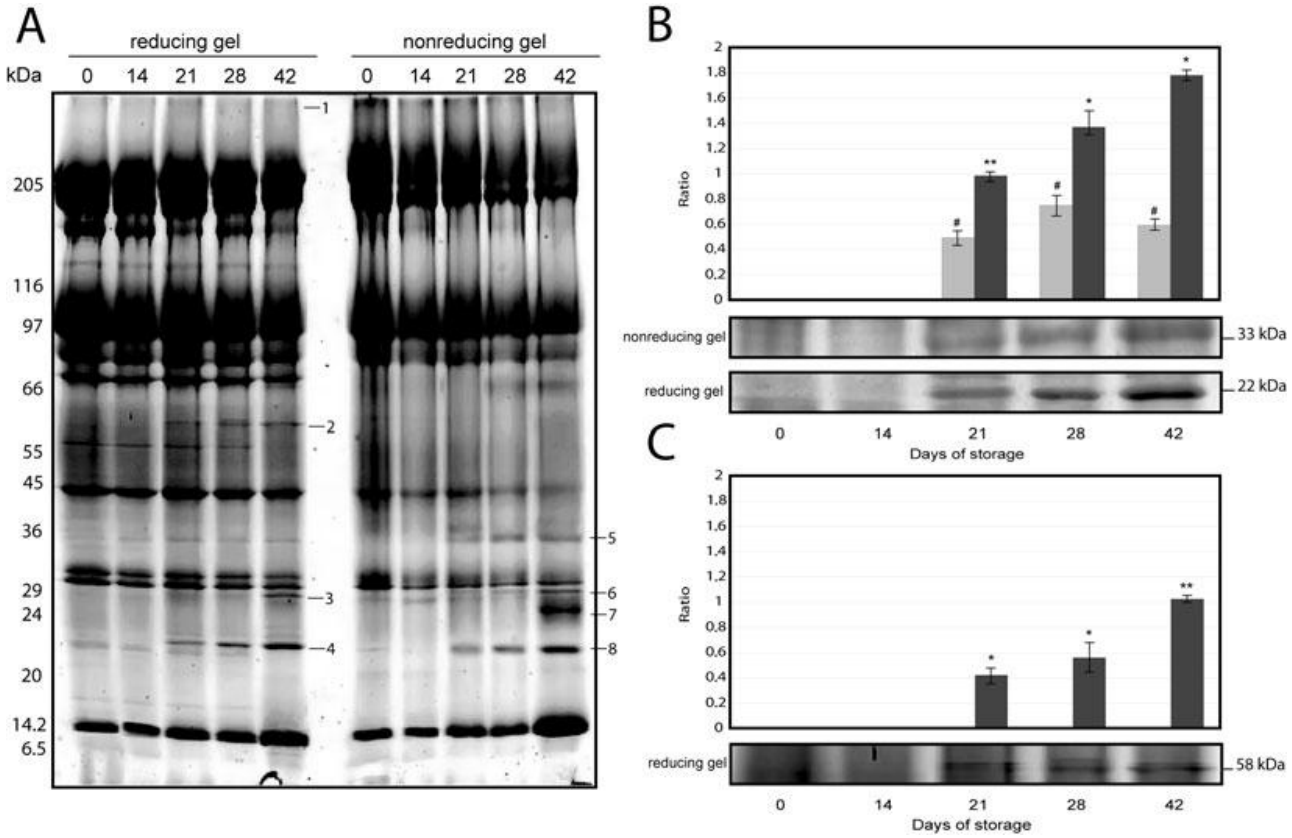

Fig. 13. Representative electrophoretic profile of membrane proteins from RBCs stored for 0, 14, 21, 28, and 42 days (A). SDS-PAGE was performed in a linear gradient (7%-14%) gel and samples treated under reducing (left side) and non-reducing (right side) conditions (molecular weight markers are shown on the left). Numbers refer to protein identified by mass spectrometry and detailed in Table 1. PrxII (B) and CAT (C) levels were measured by densitometric analysis of electrophoretic gels as ratio between each sample and the used internal controls. Data are presented as mean ± standard error (n = 3). *#$p < 0.05$. **$p < 0.01$.

The high-molecular-weight Band 1 was detected in increasing amounts toward the end of the storage period and it was present both under reducing and non-reducing conditions. This evidence suggested that band is stabilized by non-reducible linkages (such as amides or free radical–generated adducts). Tandem mass spectrometry (MS/MS) analysis (see Table 2) revealed the presence in this band of non-reducible cross-linking species of Hb with some important cytoskeletal proteins, such as spectrin, ankyrin and Band 3.

Band number (apparent M_r)*	Protein identification (*Homo sapiens*)	NCBI accession number	M_r, kDa theoretical†	Number of peptides identified by MS/MS	Sequence coverage (%)	Mascot score
1 (~500 kDa)	α-Spectrin	gil338438	282.0	17	9	779
	Ankyrin	gil178646	207.1	13	9	673
	Band 3 anion transport protein	gil4507021	102.0	10	15	513
	β-Spectrin	gil338441	247.0	4	2	215
	Hb β-chain	gil4504349	16.1	3	26	122
	Hb α-chain	gil57013850	15.2	3	23	105
2 (62 kDa)	CAT	gil4557014	59.9	23	49	1520
3 (27 kDa)	2,3-BGM	gil4502445	30.1	5	23	210
4 (22 kDa)	Prx2	gil32189392	22.0	15	42	882
5 (35 kDa)	Prx2	gil32189392	22.0	10	33	437
6 (27 kDa)	2,3-BGM	gil4502445	30.1	4	20	201
7 (25 kDa)	Hb β-chain	gil4504349	16.1	11	78	715
	Hb α-chain	gil 57013850	15.2	6	36	356
8 (22 kDa)	Prx2	gil32189392	22.0	14	38	853

* The apparent molecular masses of protein bands were estimated by comparison with standard protein markers (Fig. 1A).
† Theoretical M_r was calculated using the Expasy tool (http://expasy.org/tools/pi_tool.html).

Table 2. Mass spectrometry identification of protein bands from SDS-PAGE of ghosts prepared from stored RBCs.

Under reducing conditions, CAT band (Band 2, 59.6 kDa) was detected in ghosts only after the 21st day of storage, and its volume increased with the duration of the storage. Under non-reducing conditions, CAT band disappeared suggesting that intermolecular disulfide bonds could be involved in the membrane association. Electrophoretic gels (under both reducing and non-reducing conditions) also showed the association of the multifunctional enzyme 2,3-bisphosphoglycerate mutase (2,3-BGM) to ghost membrane (Bands 3 and 6, 29.9 kDa). The interaction appeared at the end of the storage period (42 days). Interestingly, starting from Day 21, a marked accumulation in PrxII protein was evident by reducing SDS gel analysis (Band 4, 22.5 kDa). Normalized volume of PrxII band sharply increased with storage time, reaching the 82.3% of the initial value on Day 42 (Fig. 13B). Under non-reducing conditions a new band at about 34 kDa was identified as dimeric form of PrxII protein (Band 5). Similarly to the monomeric band also the dimeric form increased approaching 42 days of storage. These data suggested that cytoplasmic PrxII protein might link to the RBC membrane in a dimeric form. Finally, storage time induced the appearance of membrane-bound Hb dimers, which can be seen as a new protein band at approximately 24 kDa in non-reducing gel (Band 7). MS/MS analysis identified in this band both a- and b-globin chains, indicating the association of Hb heterodimers due to intermolecular disulfide bonds to the RBC membrane.

3.2 Effect of atmospheric oxygen on protein migration

To evaluate the role of atmospheric oxygen on the cytoplasmic protein migration to ghost membranes, we performed RBC storage under anaerobic conditions. The SDS-PAGE profile of ghost membranes extracted from RBCs stored in the absence of oxygen showed some

important differences when compared against aerobically stored counterparts that corroborated the involvement of oxidative stress in protein migration (Fig. 14).

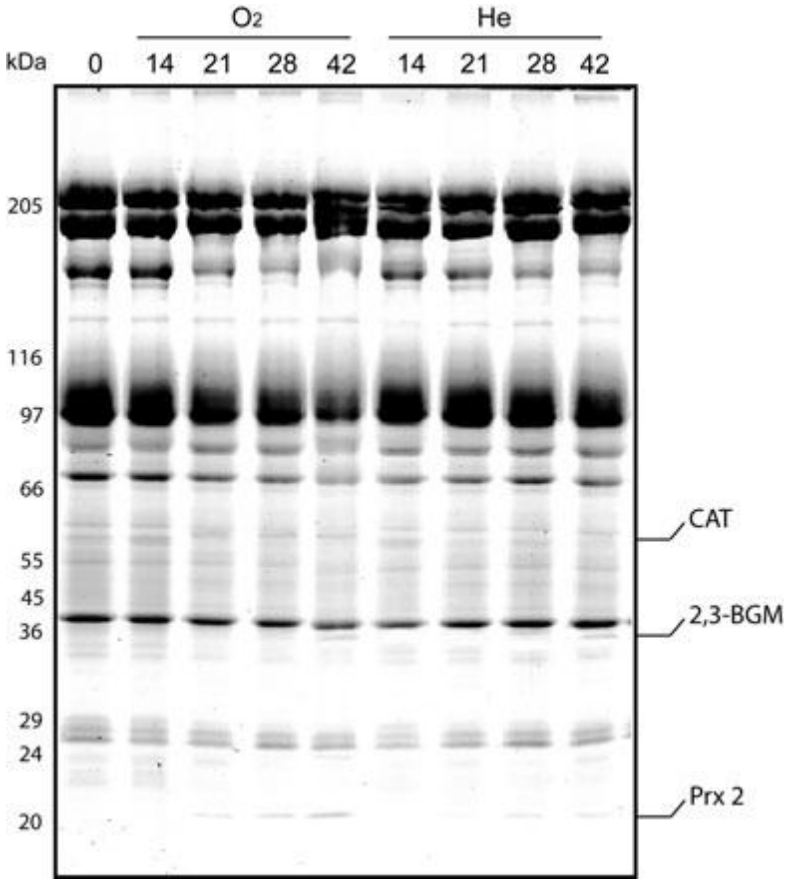

Fig. 14. Representative SDS-PAGE of RBC ghosts yielded at different storage times under aerobic and anaerobic conditions. Electrophoretic run was performed in a 9% acrylamide gel and samples were treated with reducing agent.

In particular, the CAT band disappeared completely when RBCs were stored under helium, indicating that when ROS genesis was likely to be inhibited,23 the migration of this enzyme from cytosol to membrane was suppressed. Similarly, the antioxidant enzyme PrxII showed a reduced band volume in anaerobiosis when compared to aerobic conditions, demonstrating that its membrane linkage is associated with oxidative mechanisms. In contrast, anaerobiosis did not prevent the 2,3-BGM migration to the membrane, suggesting that the process was not ROS mediated.

3.3 PrxII membrane migration: evaluation of interindividual variability.

To evaluate interindividual variability of PrxII membrane migration we achieved immunoblotting analysis under non-reducing conditions (Fig. 15A) of RBC membranes extracted from eight different RBC units after 0, 14, 21, 28, and 42 days of storage.

All RBC bags were stored following the European Council Guidelines in SAG-M at 4°C [Council of Europe, 2007] and PrxII redox forms present within the cell were locked during ghost membrane preparation by means of incubation with an alkylating agent (NEM). To remove nonspecifically membrane-bound PrxII, additional washings with 0.9% NaCl were carried out on RBC membranes before SDS-PAGE. Gels showed that PrxII linked to RBC membrane after different periods of storage, while it was never present in 0-day samples. A total of 12.5% of samples (1 RBC unit out of 8) showed PrxII monomeric and dimeric bands for the first time after 14 days of storage, 50% of samples (4 RBC units out of 8) after 21 days, and 37.5% (3 RBC units out of 8) after 25 days. For all tested units we found that PrxII was not associated to RBC membrane before 14 days of storage. In parallel, PrxII was present in cytosol almost exclusively as monomer until the 42nd day (data not shown). By using an internal control of human PrxII in the immunoblots, we performed a densitometric analysis on PrxII dimeric band to evaluate the amount of PrxII linked to the membrane. On the same day of storage we found that the amount of membrane bound PrxII was different between the 8 donor units, but in all cases this value decreased at 42 days. These data suggested that the kinetics of PrxII membrane migration changed among different donors, allowing us to speculate that migration was correlated to the antioxidant defense capacity of each individual donor. Indeed, one of the tested units (Donor 9) showed a distinguishing feature in the PrxII membrane migration process. Immunoblots for PrxII in non-reducing conditions of RBC membranes extracted from this donor showed both monomeric and dimeric band of PrxII just on Day 0 of sampling (Fig. 16A).

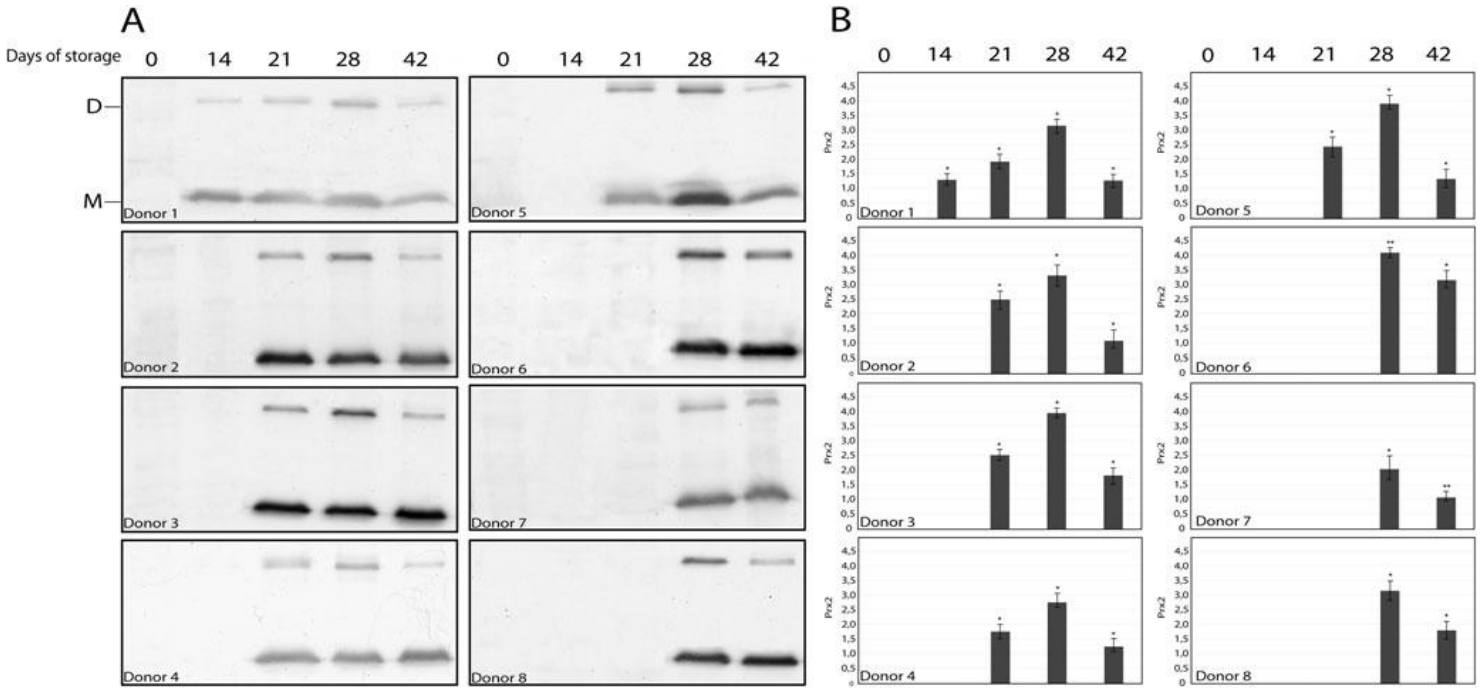

Fig. 15. PrxII immunoblots of RBC membranes prepared from RBC suspensions of Donors 1 through 8 at 0, 14, 21, 28, and 42 days of storage (A). SDS-PAGE was performed in 14% acrylamide gel under oxidizing conditions in the presence of 100 mmol/L NEM. Densitometric analysis of the immunoblots was conducted measuring the ratio between each sample and an internal control of human recombinant PrxII (B). Data are presented as mean standard error (n = 3). *p < 0.05, **p < 0.01. D = Dimer; M = monomer.

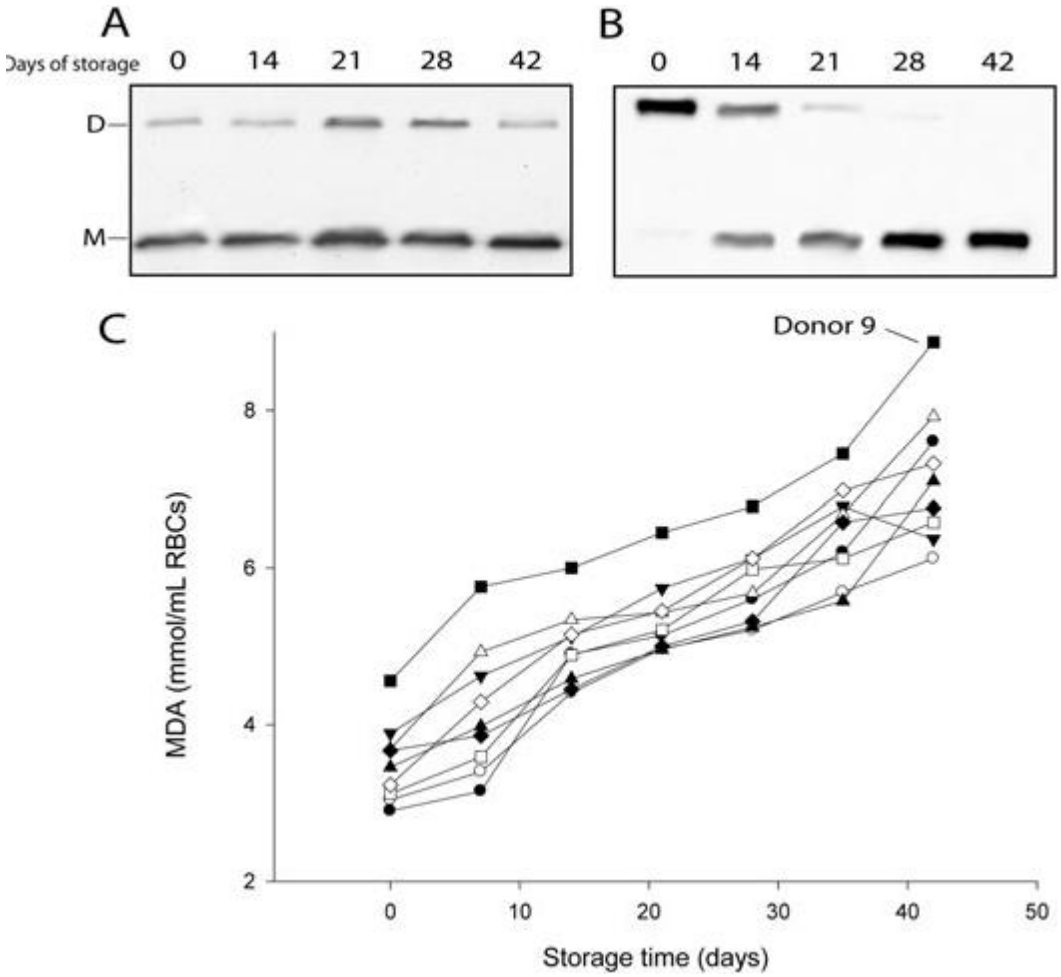

Fig. 16. PrxII immunoblots of membrane (A) and cytosolic (B) fractions from RBC suspensions of Donor 9 at 0, 14, 21, 28, and 42 days of storage. Electrophoretic run was performed in 14% acrylamide gel under non-reducing conditions in the presence of 100 mmol/L NEM. D = dimer; M = monomer. (C) RBC membrane lipoperoxidation measured as MDA level in SAGM RBC units from Donors 1 to 9. MDA values are expressed as mmol/mL RBCs. (◇) Donor 1; (□) Donor 2; (▲) Donor 3; (●) Donor 4; (▼) Donor 5; (△) Donor 6; (○) Donor 7; (◆)Donor 8; (■) Donor 9.

Also in this case, densitometric analysis revealed a marked decrease of membrane-bound PrxII dimers after 42 days of storage (data not shown). However, when cytosolic fractions from stored RBCs of this donor were probed for PrxII, an unexpected result came out (Fig. 16B). As shown, PrxII was in dimeric form on Day 0 (like the corresponding ghost sample). Furthermore, a PrxII conformational change from dimer to monomer was detected during storage time. Since it has been demonstrated that peroxiredoxins—PrxII included—have also the property to scavenge organic peroxides, such as lipid hydroperoxides [Wood *et al*., 2003], we decided to measure the RBC membrane peroxidation through the detection of MDA. Although this test is generally affected by low reproducibility due to interfering substances, it remains a useful indicator of lipoperoxidation and correlates well with the degree of oxidative stress status [Srour *et al*., 2000]. For all donor units MDA was measured and compared on 0,

7, 14, 21, 28, 35, and 42 days of blood storage (Fig. 16C). A general and gradual increase of MDA values was observed over storage, substantially correspondent to the PrxII concentrations detected until Day 28. Interestingly, MDA values in Donor 9 were the highest at all storage times, which can be accounted as evidence of basally increased RBC oxidative stress in this donor.

3.4 Native structure of erythrocyte PrxII as consequence of storage-dependent oxidative stress

In order to investigate the oxidative stress-dependent changes on native structure of human erythrocyte PrxII two conditions were examined: i) fresh erythrocytes (cells obtained immediately after blood collection) and ii) 28-day-old erythrocytes (cells obtained after 28-day storage under standard blood bank conditions). Proteomics-based approaches were applied to cytosol and ghost membranes from both these samples in order to study the oxidation and oligomerization status of erythrocyte PrxII. PrxII redox forms which are present within the RBCs were locked by means of incubation with a thiol-blocking agent (NEM) before sample preparation, so as to avoid any artifact of oxidation upon cell lysis.

3.4.1. Influence of red cell age

Since our samples (fresh and stored blood) consisted in mixtures of erythrocytes with different age, we performed a preliminary analysis on density-fractionated red cells in order to verify the contribution of old cells in determining the PrxII oxidation status. Thus, cell population was split into different age fractions according to their density and two populations were analyzed: i) fraction F1 with red cell density ≤ 1.10 containing young erythrocytes; ii) fraction F2 with red cell density ≥ 1.11 corresponding to old erythrocytes. From both fractions, membrane and cytosol were prepared and immunoblotted with anti-hPrxII antibody under non-reducing conditions (Fig. 17).

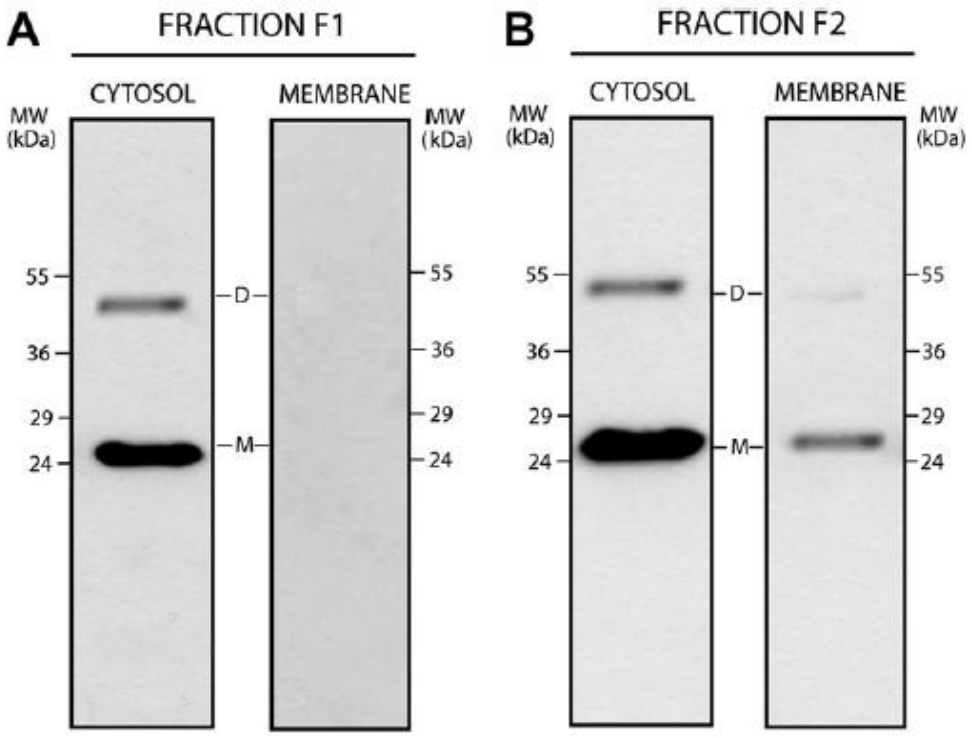

Fig. 17. Immunological detection of PrxII in cytosol and membrane of density-fractioned red blood cells at 0-day storage. Two different age RBC fractions were analyzed: (A) fraction F1 with red cell density _1.10 containing young erythrocytes, (B) fraction F2 with red cell density _1.11 corresponding to old erythrocytes. Letters D and M indicate the position of dimeric and monomeric PrxII, respectively.

In agreement with previous observations [Biondani *et al*., 2008], results demonstrated that no significant differences exist in the amount of PrxII between samples representing red cells of different mean age. In fact, although older RBC membranes contained traces of both PrxII monomer and dimer, the percentage of old erythrocyte fraction was only about 3% of the total RBCs (see Table 3), thus its contribution can be considered negligible [Alderman *et al.*, 1980].

Fraction	Density range	Percent to the total RBCs	
		0-day	28-day
F1	≤1.10	96–97%	60–70%
F2	≥1.11	3–4%	30–40%

Table 3. RBC fractionation results obtained by Percoll gradient.

During storage, there are no mechanisms to replace older RBCs, thus the ratio of young to old cells decreases in the storage bag (Table 3). By and large, these findings reinforce the choice to use whole blood as investigation material since the information obtained with the single

populations did not significantly influence the study. At the same time, we avoided excessive sample manipulation by investigating a product with important clinical implications as well.

3.4.2. Oligomeric conformation of PrxII at 0-day storage

When 0-day cytosol was resolved by Clear Native (CN)-PAGE in absence of reducing agents and detergents, Colloidal Coomassie staining indicated seven different bands (Fig. 18A, left side).

MS/MS analysis of these gel bands (Table 4, panel A) revealed the presence of the following multiprotein complexes: proteasome heterocomplex (band 1), hemoglobin ecarbonic anhydrase heterocomplex (band 2), CAT homotetramer (band 3) and the oxidized protein hydrolase homocomplex (band 4). PrxII oligomers were detected in four bands at ~440 kDa (band 3 where PrxII comigrated with CAT), ~140 kDa (band 5), ~100 kDa (band 6) and ~67 kDa (band 7). In the case of RBC ghost membranes, Blue Native (BN)-PAGE electrophoretic systems were adopted rather than CN-PAGE to ensure a higher band resolution.

Four major gel bands were distinguishable by BN gels of membrane ghosts extracted from fresh blood (Fig. 4A, right side). MS/MS analysis of band 10 at 700 kDa revealed the presence of a HMW flotillin homocomplex. Erythrocyte membrane protein band 3 was identified in two bands (440 kDa, band 20; 110 kDa, band 40) supporting the existence of a tetrameric and monomeric conformation state [Low, 1986]. No stable band 3 protein dimers were detected. Finally, human erythrocyte glucose transport protein (GLUT1) was identified as a possible tetramer [Zottola *et al*., 1995] in-gel band 30 at about 200 kDa. Thus, in agreement with previous studies [Rocha *et al*., 2008; Rinalducci *et al*., 2011, Rocha *et al*., 2010], no PrxII was found in control RBC membranes (0-day storage). To validate MS identifications, we performed western blotting analysis with an anti-hPrxII antibody on native gels. Such an assay confirmed the presence of four different PrxII oligomers in RBC cytosol and the complete absence of any PrxII complex in the ghost membranes (Fig. 18B).

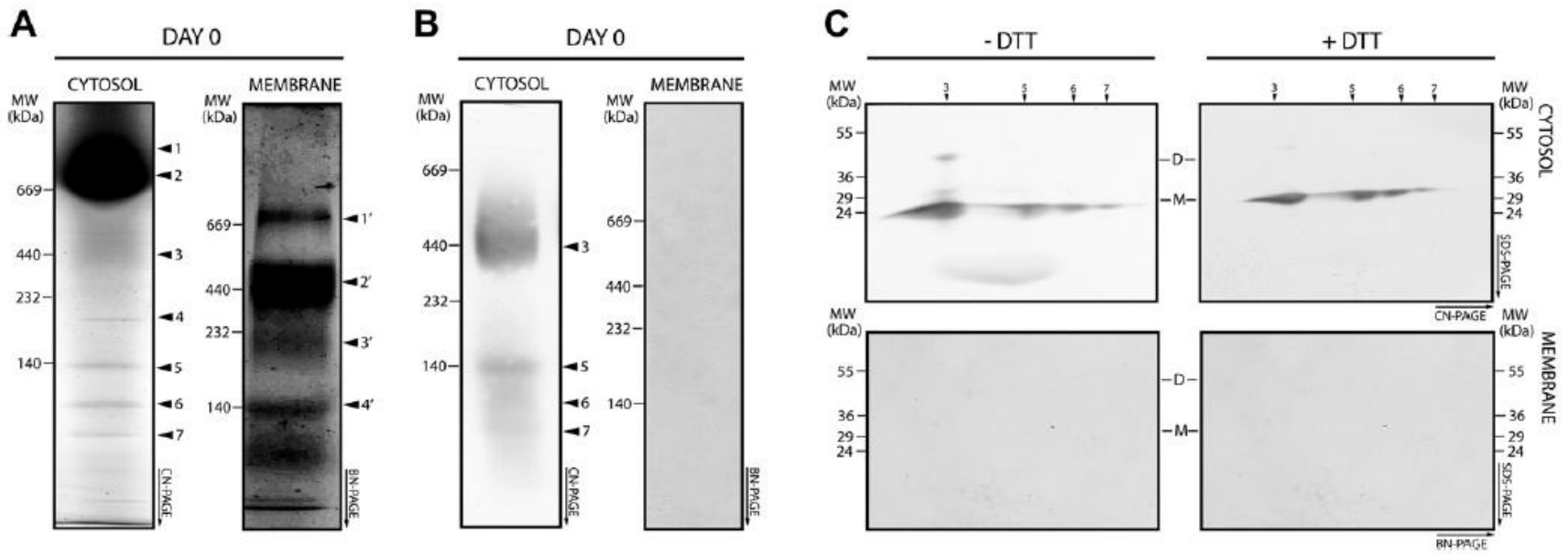

Fig. 18. Detection of PrxII oligomeric conformation at 0-day storage. Erythrocyte cytosol and membranes, obtained from RBC concentrates after 0 day of storage were separated by 1D CN- and BN-PAGE, respectively (A). Native gels were also blotted on polyvinylidene difluoride membrane and probed with an anti human reduced PrxII antibody (B). Second dimension gels in reducing and non-reducing conditions were also run for cytosol and membrane samples (C). D, PrxII dimer. M, PrxII monomer. One representative gel from the other eight with similar results is presented.

Multiprotein complex	(A) 1D CN-PAGE				(B) 1D BN-PAGE			
	Apparent molecular mass[a] (kDa)	Band number	MS/MS identification		Apparent molecular mass[a] (kDa)	Band number	MS/MS identification	
			Protein name	Sequence coverage			Protein name	Sequence coverage
Proteasome heterocomplex	~900	1	Proteasome alpha 7 subunit Proteasome beta 1 subunit	19% 15%	~1000	5′	Proteasome alpha 7 subunit Proteasome beta 1 subunit	19% 10%
Carbonic anhydrase-hemoglobin heterocomplex	670	2	Hemoglobin subunit beta Hemoglobin subunit alpha Carbonic anhydrase I	69% 25% 16%	Absent	Absent	Absent	Absent
Flotillin homocomplex	Absent	Absent	Absent	Absent	700	1′	Flotillin I Flotillin II	47% 39%
Peroxiredoxin II–catalase heterocomplex	440	3	Catalase Peroxiredoxin II	29% 34%	440	2′	Catalase Peroxiredoxin II	30% 19%
Band 3 homocomplex	Absent	Absent	Absent	Absent	440	2′	Erythrocyte membrane protein band 3	11%
Oxidized protein hydrolase homocomlplex	200	4	Oxidized protein hydrolase	18%	Absent	Absent	Absent	Absent
Glucose transport glycoprotein homocomplex	Absent	Absent	Absent	Absent	200	3′	Glucose transporter glycoprotein	16%
Peroxiredoxin II homocomplex	140	5	Peroxiredoxin II	34%	Absent	Absent	Absent	Absent
Band 3	Absent	Absent	Absent	Absent	110	4′	Erythrocyte membrane protein band 3	20%
Peroxiredoxin II homocomplex	100	6	Peroxiredoxin II	34%	Absent	Absent	Absent	Absent
Peroxiredoxin II homocomplex	67	7	Peroxiredoxin II	34%	Absent	Absent	Absent	Absent

Table 4. Multiprotein complexes identified on native gel systems. [a] The apparent molecular masses of protein complexes separated on CN and BN-PAGE were estimated by their comparison with the molecular masses of standard protein complexes (GE Healthcare).

To evaluate the nature and composition of these polymeric complexes, we run second dimensions of CN-PAGE under denaturing conditions in the presence or absence of reducing agents and immunoblotted with antibodies to PrxII (Fig. 18C). Interestingly, cytosol data showed that the highest molecular weight PrxII complexes (band 3) consisted of PrxII oligomers containing both the PrxII reduced monomer and oxidized dimer. Considering the co-migration in this band of the CAT homotetramer (expected molecular weight of w232 kDa), and the theoretical mass of a PrxII monomer (about 22 kDa), it is reasonable to assume that gel band 3 might represent the PrxII decameric state. On the contrary, bands 5, 6 and 7 resulted to be formed only by PrxII monomers, suggesting that these are homo-polymeric complexes which consist of variable copies of PrxII subunits. No spots were detected in two dimensional gels of ghost membrane samples.

3.4.3 Oligomeric conformation of PrxII at 28-day storage

In comparison to 0-day gels, CN-PAGE of cytosol samples extracted after four weeks of storage showed a smearing of both CAT-PrxII and hemoglobinecarbonic anhydrase heterocomplex bands (Fig. 3A), moreover the apparent molecular weight of CAT-PrxII band was incremented of about ~40 kDa (see later). No significant changes in PrxII oligomer band intensity were detected, with the exception of 0-day gel band 6 which disappeared completely (oligomer at 100 kDa).

On the contrary, in BN-PAGE of 28-day ghost membranes we recognized five bands, all of them containing the same complexes revealed in 0-day samples, with the exception of band 50 (~1000 kDa) which was newly-detected and identified by MS/MS as proteasome heterocomplex (see Table 2, panel B). According to Coomassie blue stained gels, immunoblotting with an anti-hPrxII antibody identified only three PrxII oligomer complexes in the cytosol, at ~480, 140 and 67 kDa of molecular weight (Fig. 19B). Surprisingly the CAT-PrxII heteropolymeric complex was detected also in ghost membranes. This evidence together with the previously mentioned appearance in membrane of proteasome complex, indicated a recruitment of these two important protein complexes to the erythrocyte membrane under oxidative stress conditions. Second dimension denaturating gels under reducing and non-reducing conditions were immunoblotted for PrxII (Fig. 3C), as performed for 0-day samples.

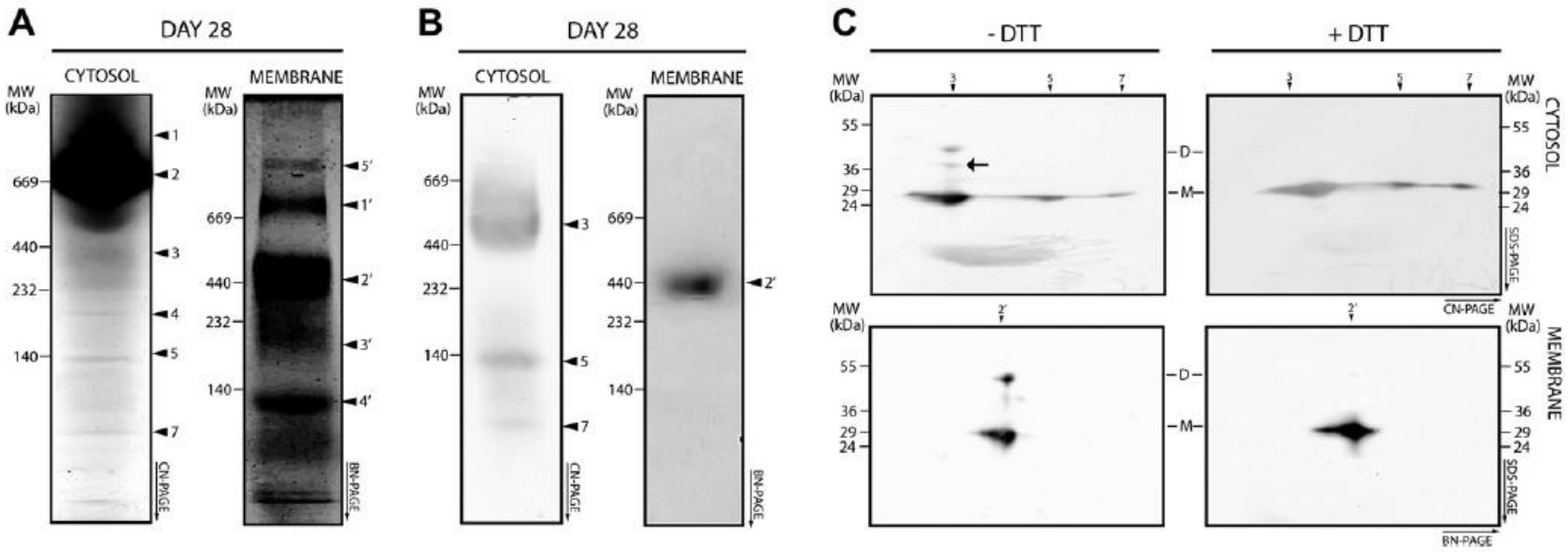

Figure 19. Detection of PrxII oligomeric conformation at 28-day storage. Erythrocyte cytosol and membranes, obtained from RBC concentrates after 28 days of storage were separated by 1D CN- and BN-PAGE, respectively (A). Native gels were also blotted on polyvinylidene difluoride membrane and probed with an anti human reduced PrxII antibody (B). Second dimension gel in reducing and non-reducing conditions were also runned for cytosol and membrane samples (C). Letters are as in Fig. 18. The arrow indicates the position of PrxII cross-linked to hemoglobin chain. Results shown are representative of experiments done in replicates (n = 9).

Cytosol results from non-reducing gels showed that the complex at 480 kDa contained three different spots recognized by the anti-hPrxII antibody. Mass spectrometric analysis confirmed the identification of PrxII in the gel bands at 24 and 50 kDa, respectively as the monomeric and dimeric form. On the contrary, the intermediate band at 40 kDa (Fig. 19C, spot indicated by an arrow) was identified as PrxII cross-linked to hemoglobin chain (data not shown). This spot disappeared in the presence of reducing agent, thus PrxII and Hb may be bound via disulfides. Finally, native PrxII oligomers at 140 and 67 kDa resulted to be formed exclusively by monomer subunits. Data from second dimension immunoblotted gels of 28-day ghost membranes showed that the CAT-PrxII heterocomplex at 440 kDa contained PrxII both as monomer and dimer, similarly to cytosol. However, a significant increment of the PrxII dimeric state was evident in the oxidized membranes with respect to the cytosol fraction. To further investigate the PrxII oxidative state, we evaluated PrxII cysteine sulfinic acid (Cys-SO_2H) and cysteine sulfonic acid (Cys-SO_3H) by probing erythrocytes with antibodies to overoxidized PrxII. Overoxidized forms of PrxII were not detectable in any samples (data not shown).

3.5. Peroxidase activity

Since the oligomeric structure of PrxII is strictly correlated with its function, peroxidase activity staining of CN and BN gels was carried out as described in Materials and methods. Hydrogen peroxide was used as substrate and 1.15 mM sodium azide was added to the reaction mixture in order to inhibit CAT activity. Moreover, to avoid any interference due to Hb pseudoperoxidase activity, gel strips were incubated with the Drabkin's reagent (potassium ferricyanide/potassium cyanide) to convert Hb to cyanomethemoglobin before assaying. In these conditions, only cytosol PrxII oligomers at 440 and 140 kDa exhibited peroxidase activity both at 0 and 28 days of blood storage (Fig. 20A). Membrane PrxII oligomers at 440 kDa resulted to be active as well in catalyzing hydrogen peroxide reduction (Fig. 20B). Moreover, due to the capability of peroxiredoxins to scavenge also organic peroxides we tested the reactivity of PrxII membrane complexes toward t-butyl hydroperoxide, in order to determine their eventual involvement in the mechanism of defense against lipid peroxidation. Interestingly, the assay performed on native gels of 28-day stored ghosts demonstrated a positive reaction (Fig. 20C).

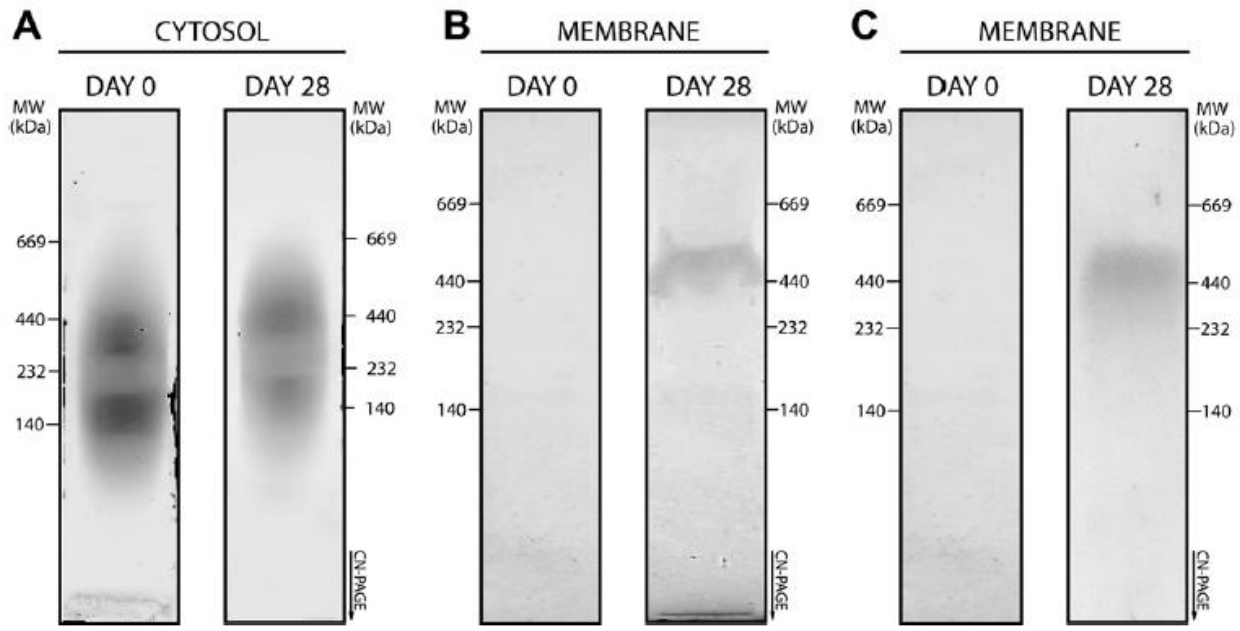

Figure 20. PrxII activity staining on 1D native PAGE. Erythrocyte cytosol (A) and membranes (B), obtained from RBC concentrates after 0 and 28 days of storage were separated by first dimension native PAGE and peroxidase activity staining against H2O2 was carried out as described in materials and methods. In addition, activity staining for organic peroxides was also tested in membrane samples (C). All samples used for enzyme activity assays were prepared without NEM.

Chapter 4

Discussion

4.1 PrxII as a candidate biomarker to test oxidative stress levels of stored red blood cells under blood bank conditions

Oxidative stress is a common feature of RBC storage lesions and it has been postulated that the oxidative events occurring in stored RBCs may be correlated with the risk of post-transfusion complication [Zimrin and Hess, 2009; Ho *et al*., 2003; Tinmouth *et al*., 2006; Hess, 2010]. Thus, the knowledge of both donor's antioxidant defense capacity and the grade of oxidative stress during storage time may represent the key link to improve the quality of stored RBCs. In this context, discovery of biomarkers to evaluate the grade of oxidative stress, both in blood donors and during storage time, can play a key role. RBC membrane proteins represent a primary target of the various pathologic influences of storage; therefore, they are accounted as the major determinants of the clinical outcome of RBC storage lesion. For example, RBC deformability is tightly connected to the structural and functional integrity of the RBC cytoskeleton and its storage-related loss may account for impaired microvascular oxygenation after transfusion [Ho *et al*., 2003]. On the other hand, recent advances in the study of RBC storage lesions supported the oxidation of the membrane as an important variable of the pathophysiology of stored cells [Kriebardis *et al*., 2006]. Thus, the characterization of possible alterations on RBC membrane composition, documented as storage-related membrane recruitment of preferential cytosolic proteins, was the aim of our study with the attempt to present potential biomarkers of oxidative stress in human RBCs. Our results demonstrated that four cytosolic proteins, namely, Hb, 2,3-BGM, CAT, and PrxII, migrated to RBC membrane during storage time. Association of Hb with the membrane is a well-known phenomenon.

Indeed, previous studies have shown increased amounts of Hb in ghosts after prolonged storage [Bosman *et al*., 2008; Kriebardis *et al*., 2007; Wolfe *et al*., 1986]. Our results clearly confirmed the presence of non-reducible cross-linking species of Hb with the main cytoskeletal proteins such as spectrin, ankyrin, and Band 3. These high-molecular-weight bands, representing ROS-induced pathologic multimers, were evident after 21 days of storage and became prominent approaching the end of the storage period. 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], although it can also be reproduced in vitro by treating normal RBCs with hydrogen peroxide [Snyder *et al*., 1985]. In any case, its migration to the membrane represents an aging phenomenon. Similarly, the individuation of 2,3-BGM in the RBC membranes appears to be an aging-related process, likely due to a preparation of RBCs toward disposal of damaged

proteins through vesiculation, in a sort of self-protective mechanism [Willekens *et al*., 2008; Bosman *et al*., 2008]. Accordingly with this hypothesis, the 2,3-BGM enzyme has been recently detected in microparticles originating from 42-day-stored RBCs [Bosman *et al*., 2008; Rubin *et al.*, 2008]. However, its late migration into membrane cannot explain the drop of 2,3- DPG during the storage of RBCs for transfusion, since it is almost completely lost just after 2 weeks of storage [Hogman *et al*., 2006]. CAT is a cytosolic antioxidant enzyme, but it has been reported that metabolically stressed RBCs showed calcium-dependent accumulation of membrane-bound CAT [Allen *et al*., 1977], suggesting that its presence in membrane can represent an optimal protective location in case of stress. Concordantly, our study demonstrated a storage time– dependent increase of CAT in the RBC membrane, which is detected on Day 21 during the storage period. Several mechanisms may be proposed to explain this oxidative stress–related increase of CAT membrane binding. First, the bound enzyme has an acidic isoelectric point (pI = 5.4) on exposure to oxygen [Mörikofer-Zwez *et al.*, 1969]; hence, ionic binding to the membrane after increased intracellular calcium accumulation, as typically occurs in old RBCs [Romero and Romero., 1999], may play a role. Although the data about the increased Ca2+ permeability in coldpreserved RBCs are still controversial [Wiley *et al*., 1982; McNamara and Wiley, 1987], this cannot be our case because RBCs were stored in SAGM AS. Like Hb, CAT contains cysteine residues and demonstrates micro heterogeneity, possibly related to sulfhydryl oxidation, so that membrane binding by sulfhydryl groups may represent a second possibility. To this end, our study revealed that the electrophoretic band corresponding to CAT disappeared under non-reducing conditions suggesting a possible involvement of intermolecular disulfide bonds in this membrane association. Finally, oxidative stress may alter the properties of a typical membrane protein, such as spectrin or Band 3, so as to increase its tendency to bind CAT.

Human RBC PrxII is a 2-cys peroxiredoxin, with thiol-dependent H_2O_2 scavenger activity [Wood *et al*., 2003]. It resides in the cytoplasm as dimers and decamers in a dynamic equilibrium [Kristensen *et al*., 1999]. 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]. PrxII has been shown to bind Hb to prevent its oxidation [Stuhlmeier *et al*., 2003], either as hydrogen peroxide scavenger or as chaperone-like protein [Low *et al*., 2008]. However, Rocha and co-workers [Rocha *et al*., 2009] recently demonstrated that oxidized Hb and PrxII membrane linkages appear to be independent processes although both resulting from oxidative stress. Interestingly, we showed that under non-reducing conditions PrxII was

significantly dimerized in agreement with previous studies demonstrating the high sensitivity of this protein to oxidation by H_2O_2 [Low *et al*., 2007]. Moreover, a sharp increase of the PrxII dimer was observed with storage duration, starting on Day 14. The atypical presence of each of these four proteins in the RBC membrane could theoretically represent an oxidative stress marker to be used in future quality control (QC) programs in blood banking. However, some of them are most probably to be excluded as biomarkers of oxidative stress damage in such programs. In fact, although membrane association of Hb is correlated to ROS activity, its detection in control ghosts is anything but occasional and its increase is not always linear with respect to the level of oxidative stress [Kriebardis *et al*., 2007]. For these reasons it cannot be objectively measured and evaluated as indicator of oxidative injury during the storage period. Equally, 2,3-BGM can be excluded as candidate biomarker due to its late accumulation in the membrane of stored RBCs (after 42 days) and because its presence was observed also under anaerobic conditions, indicating that its migration is not associated with oxidative mechanisms. On the contrary, both CAT and PrxII were strongly reduced when RBCs where stored under helium and both could be proposed as oxidative stress biomarkers in RBCs. The presence of these cytosolic enzymes on stored RBC membranes can be considered a distinctive feature with intrinsic qualities as high specificity and stability, which allow objective measurements of oxidative stress status. Both enzymes were not present in control samples (0 days of storage) and their migration to the membrane progressively increased during storage time. At any rate, we suggest PrxII as biomarker for a series of aspects. First, its membrane-linked amounts are notably higher than CAT and this makes its detection easier. In fact, to function as a suitable biomarker it is critical for the candidate protein: 1) to be present at sufficiently high concentrations so as to represent a significant product, under the same conditions at different times; 2) to be a major product of oxidative damage that may be significant for diagnostic purposes; and 3) to be easily detectable and measurable by means of assays that are specific, sensitive, and reproducible to be used as a diagnostic tool [Dalle-Donne *et al*., 2006]. Second, the dimeric conformation of PrxII gives important information on the oxidative state of the protein indicating a reduced H_2O_2 scavenger activity. Moreover its estimation can exclude possible contaminations from cytosolic PrxII portion because dimer has never been detected in control RBC ghosts [Rocha *et al*., 2009; 2010]. Indeed, in cytosol PrxII exists in a redox-linked oligomerization state with the reduced enzyme (monomer) forming the $(\alpha 2)_5$ decamer exclusively. Cysteine oxidation induces dimerization and decamer dissociation, although under normal conditions dimer is quickly reduced to monomer by NADH-dependent enzymatic system and its $t_{1/2}$ is very short. Thus, the more stable and

predominant form is the decamer in which PrxII exists in monomeric form [Wood *et al.*, 2002]. On the other hand, the amount of membrane-bound PrxII increases during the storage indicating that either there is a continuous migration of PrxII from cytosol to membrane or PrxII is oxidized to dimer over time until vesiculation prevails approaching 42 days, as shown in previous reports [Bosmann *et al*., 2008; Antonelou *et al*., 2010]. Concomitantly, it should be hypothesized that the NADH-dependent enzymatic system for dimer reduction does not work at the membrane level. Further investigations are needed to clarify the native conformation of PrxII in the membrane as well as the activity of PrxII in the cytosol-membrane and possibly the mechanism of its linkage in oxidative stress conditions.

To support the proposal of PrxII as possible biomarker of oxidative stress in blood banking, we evaluated its migration to RBC membrane in terms of biologic variability. Our findings showed a time-dependent linkage of the PrxII dimer to the membrane, ranging from Day 14 to Day 28.Moreover, the variability of PrxII binding to RBC membrane at different times may suggest that each donor has a different susceptibility to oxidative stress due to individual antioxidant activity level. Indeed, we found a donor showing PrxII in the membrane just after blood drawing. This sample showed a higher MDA level demonstrating an advanced RBC membrane lipoperoxidation. Although further confirmations are needed, this is preliminary evidence that in this donor the RBC antioxidant defense system is ostensibly compromised leading to a more pronounced condition of RBC oxidative stress during storage. In conclusion, our data indicate PrxII as a candidate biomarker for RBC oxidative injuries under blood bank conditions, possibly to be utilized in future blood component QC programs, provided routinely applicable tests are developed and inter individual variability is duly taken into consideration as a possible bias. Furthermore, testing donors' blood susceptibility to oxidative injuries on Day 0 could also prove helpful in identifying donors whose blood could develop earlier storage lesions. An experimental study is under way at our institutions with the aim to associate blood donors presenting early RBC oxidative damage with specific standard anamnestic findings. Finally, PrxII might be used in correlation with MDA testing to improve the grade of reproducibility and specificity of the latter, which could be revalued as a simple test to measure membrane peroxidation as a biomarker of RBC oxidative stress status.

4.2 Oxidative stress-dependent oligomeric status of erythrocyte peroxiredoxin II (PrxII) during storage under standard blood banking conditions

Prxs were discovered relatively recently and little attention has been given to their antioxidant role in human erythrocytes, especially under conditions of acute oxidative stress. Our work is

the first investigation on the redox-dependent PrxII oligomerization in erythrocytes under basal and storage conditions, where fresh blood represents a partially reduced system, while 28-day stored blood constitutes an oxidized environment. Findings here reported confirmed that erythrocyte PrxII shows oxidative stress-related conformational changes which are typical and distinctive with respect to other cells. This is not surprising because RBCs are particularly exposed to oxidative hazard due to their specific role as oxygen carriers [Rice-Evans, 1990]. We observed at least four different oligomers of PrxII both in fresh blood and after 28 days of storage under standard blood bank conditions. Considerable evidence supports the hypothesis that the normal aging process of RBCs is accelerated during storage [Bosman and Kay, 1988; Lion *et al*., 2010; Hess, 2010], and existing data bring into question the role of H_2O_2 as a major mediator of RBC storage lesions [Józwik *et al*., 1997; Korgun *et al*., 2001; Dumaswala *et al*., 1999; 2000]. Nevertheless, peroxidative processes in stored blood may affect RBCs to a greater extent than is generally believed, especially now that Prxs were demonstrated to play a key role in erythrocyte defense against oxidative insult [Johnson *et al*., 2005; 2010; Lee *et al*., 2003]. On other hand, these unique redox properties of PrxII in erythrocytes were assessed to date only in total cell extracts and under in vitro stress conditions (addition of H_2O_2 with concentrations up to 200-300 mM) [Low *et al*., 2007], without looking at the native oligomeric complexes. Our study aimed instead to monitor oxidative stress-dependent structural and functional switching of human red cell PrxII both in cytosol and ghost fractions, especially in the light of recent observations for a membrane recruitment of PrxII [Rocha *et al*., 2008; Antonelou *et al*., 2010; Rinalducci *et al*., 2011; Rocha *et al*., 2010].

4.2.1 Prx in partially reduced system (0-day storage)

In fresh blood we found that cytosolic red cell PrxII exists as at least four different homo-polymeric complexes consisting of variable copies of its subunit, whereas no PrxII complexes were detected in ghost membranes. The highest molecular weight PrxII protein complex (440 kDa) derived from the association between tetrameric catalase (232 kDa) and decameric PrxII. Such a partnership has never been documented before, although binding of 2-Cys Prxs to other proteins have been shown in diverse systems [Caporaletti *et al*., 2007; Lee *et al*., 2001; Jönsson *et al*., 2008]. Thus, if on the one hand the interaction itself might not surprise, on the other hand the specific interaction with catalase acquires an intriguing singularity due to the fact that catalase, Prx and glutathione peroxidase represent the major erythrocyte

players in detoxifying peroxides. Interestingly, the 440 kDa complex showed to contain both reduced and oxidized (disulphide-linked dimers) PrxII oligomers (see Fig. 18C). It is largely documented that typical 2-Cys Prxs undergo redox-sensitive oligomerization [Hall *et al*., 2009; Barranco-Medina *et al*., 2009]. In particular, these studies revealed that the reduced or overoxidized forms of the enzyme favored the decameric state, whereas the disulphide-bonded forms existed predominantly as dimers. Despite the reported instability of the disulphide-linked form of the decamer, Wood *et al*. succeeded in trapping this important intermediate from bacteria [Wood *et al*., 2002]. Consistently with these findings, our data demonstrated the existence of stable oxidized decameric complexes in cytosol of control human erythrocytes (band 3, Fig. 18C). This result was unlikely due to a contamination of old red cells since the investigation extended to density-fractionated erythrocytes showed that the contribution of the older population to the total RBCs was negligible. Interestingly, only monomer subunits participated to the structural organization of the 140, 100 and 67 kDa complexes (bands 5-7, Fig. 18C). It worthwhile to mention that a similar PrxII complex of about of 159 kDa was detected very recently by native electrophoresis gel in human erythrocytes and authors suggested this as an octameric form [Wang *et al*., 2010], analogously to the AhpE protein of Mycobacterium tuberculosis [Li *et al*., 2005]. Nonetheless, no crystallographic evidence supports this assumption. Due to the precise link existing between oligomerization status and catalytic activity of the Prx enzyme, we verified the peroxidase activity of separated native complexes and we demonstrated its detection only in the 440 and 140 kDa bands. Thus, these two aggregation states are both catalytically active in decomposing hydrogen peroxide but may solve anyhow different roles, especially if we consider the tight association of the decamer with catalase and its different redox state (i.e., presence of disulphide-linked dimers). Many of the previously described interactions between Prxs and other proteins depend on specific redox-dependent conformational states of the binding partners. This fact supports the view that Prxs function as redox sensors or regulatory hub proteins linking cellular redox information to functional switches. The reduced form is the active status as peroxidase, which, following thiol oxidation by the peroxide substrate, is regenerated by reductants such as thioredoxins in erythrocytes. At physiologically relevant concentrations, it may be hypothesized that, when more active sites in the decamer form disulphide bonds, the instability of the complex reaches a critical point and the decamer breaks down into free dimers. However, no free dimers were revealed in the erythrocyte cytosol even after oxidative conditions such as blood banking conditions (see next paragraph).

4.2.2 Prx in oxidized system (28-day storage)

Under oxidative stress conditions (28-day storage), the PrxII aggregation state at 100 kDa in the cytosol disappeared and the CAT-PrxII 440 kDa hetero-oligomeric complex is converted to a higher molecular weight complex (band 3 at 480 kDa, Fig. 3B). In agreement, previous observations have documented that strong oxidization tends to freeze the decamer, thereby enhancing the propensity of PrxII to associate into higher order aggregates [Barranco-Medina *et al.*, 2009; Moon *et al*., 2005; Meissner *et al*., 2007]. Our results showed that cross-linked species of PrxII and Hb participate in the composition of this complex. Interestingly, Prx has been already shown to link to Hb [Johnson *et al*., 2005; Stuhlmeier *et al*., 2003]. As mentioned in the previous paragraph, no accumulation of free dimers was observed under oxidizing conditions. This evidence may be explained by an efficient and rapid regeneration system, however Low et al. demonstrated a slow PrxII turnover in human erythrocytes due to the low Trx-reductase level [Low *et al*., 2007], with consequent PrxII dimer accumulation following endogenous production of H_2O_2. Clarification of the apparent discrepancy may be found by hypothesizing that following the increase of intracellular reactive oxygen species, such as during storage, PrxII accumulates as dimer in the membrane. Ghosts of 28-day stored erythrocytes contained the same 440 kDa hetero-oligomeric complex formed by catalase homotetramer and PrxII decamer and detected in 0-day cytosol sample. Binding of PrxII to the RBC membranes has been previously described under oxidative stress conditions [Rocha *et al.*, 2008; 2010; Antonelou *et al.*, 2010; Rinalducci *et al*., 2011], however its native conformation as well as its function have never been investigated so far. The characterization of this complex by second electrophoretic dimension (Fig. 19C) showed the presence of both disulphide-linked PrxII dimers and monomer subunits, with a larger percentage of dimers. The increased rate of the dimer in 28-day stored RBC membranes suggests a possible compartmentalization of PrxII oxidized forms. On the other hand, our findings with membrane-bound PrxII also raised the possibility that oxidation to the disulphide could serve as a regulatory switch for other PrxII attributed roles, such as activator of Kþ efflux via Gárdos channels [Moore *et al*., 1991; Plisker *et al*., 1992], or chaperonin [Jang *et al*., 2004]. In fact, Plishker *et al*. have found that stimulation of K^+ transport in erythrocytes induced membrane association of high-molecular-weight oligomers of PrxII, and that these oligomers consisted of disulfide-linked dimers [Moore *et al*., 1997]. Although storage under blood bank conditions has been associated with potassium intracellular loss [Bennet-Guerrero *et al*., 2007], our assays for peroxidase activity of membrane-linked PrxII oligomers showed ability

to scavenge hydrogen peroxide. This fact seems to exclude alternative roles for the PrxII membrane complexes, at least during the first days of the storage. Interestingly, the interrelated capacity of PrxII in scavenging organic peroxides opens the possibility for a defense role of these membrane complexes against lipid peroxidation.

Through proteomics tools including native gel electrophoresis, immunoblotting and mass spectrometry, we provided new insights into the oxidation state of PrxII in human erythrocytes both in normal and stress conditions. We hereby demonstrated for the first time the existence of at least four stable aggregations states of red cell PrxII. In particular, PrxII complexes at 140, 100 and 67 kDa showed to contain variable copies of its monomeric subunit. Among these, only the 140 kDa form, probably corresponding to the heptamer, showed peroxidase activity. An active hetero-oligomeric complex composed by tetrameric catalase and decameric PrxII at 440 kDa has also been discovered. This complex migrates from cytosol to membrane following oxidative insult, suggesting PrxII as an effective redox-sensitive regulator in storage lesions. Moreover, the same oligomer was shown to be composed of both reduced and oxidized (i.e., containing disulphide-linked dimers) PrxII decamers. Thus, despite the generally reported instability of the disulphide linked form of the PrxII decamer, human erythrocytes were shown to contain this important intermediate also in normal conditions (fresh blood). Our findings demonstrate how oxidative stress governs oligomerization and structural transitions in Peroxiredoxin II which are associated to functional roles in protecting red cell membranes against storage-induced damages. A scheme showing the redox behavior of PrxII oligomers in human RBCs, as inferred by this work, is proposed in Fig. 21.

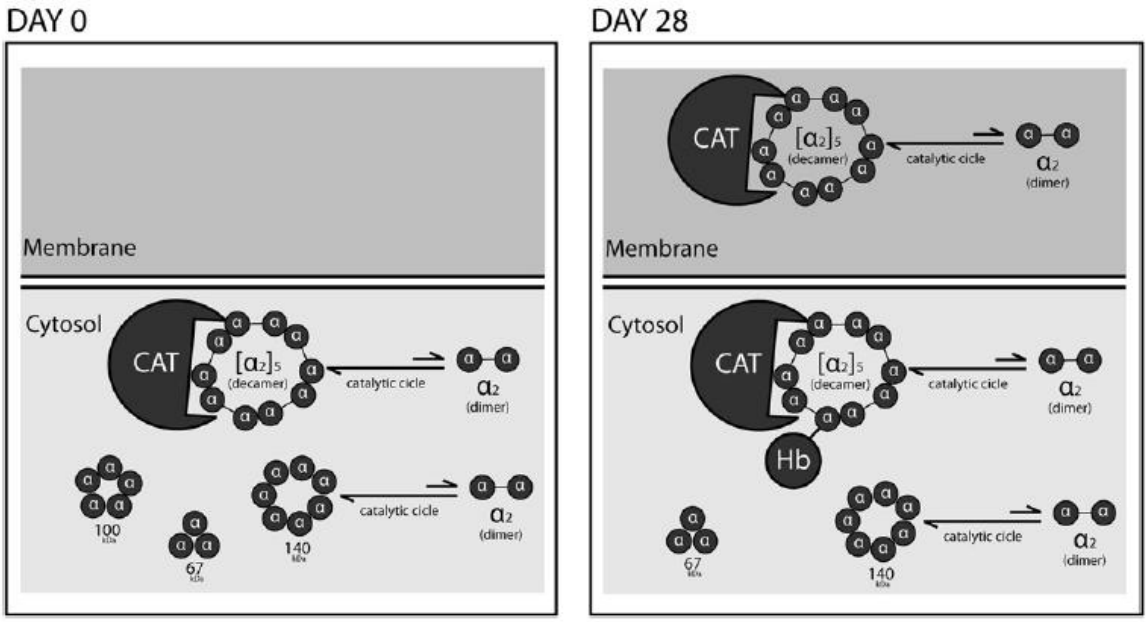

Figure 21. Proposed scheme of the redox transitions of PrxII. Schematic representation of the proposed redox behavior of PrxII in cytosol and membrane ghosts when human erythrocytes are collected from fresh blood (partially reduced system) and after 28 days of storage (oxidized environment). Number of subunits in each complex has been theoretically calculated on the basis of molecular weights on native gels and should be considered as approximate.

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