Year : 2014

Proteomics of oxidative lesions occurring during transfusion-purposed storage of erythrocyte concentrates

Julien Delobel

Centre de Transfusion Sanguine

PROTEOMICS OF OXIDATIVE LESIONS OCCURRING DURING TRANSFUSION-PURPOSED STORAGE OF ERYTHROCYTE CONCENTRATES

Thèse de doctorat ès sciences de la vie (PhD)

présentée à la

Faculté de biologie et de médecine
de l'Université de Lausanne

par

Julien DELOBEL

Master en protéomique de l'Université des Sciences et Technologies de Lille 1

Jury

Prof. Margot Thome Miazza, Président
Prof. Jean-Daniel Tissot, Directeur de thèse
Prof. Michel Duchosal, expert
Prof. Nicolas Fasel, expert
Prof. Beat Riederer, expert
Dr. Philippe Tacchini, expert

Lausanne 2014

Centre de Transfusion Sanguine

PROTEOMICS OF OXIDATIVE LESIONS OCCURRING DURING TRANSFUSION-PURPOSED STORAGE OF ERYTHROCYTE CONCENTRATES

Thèse de doctorat ès sciences de la vie (PhD)

présentée à la

Faculté de biologie et de médecine
de l'Université de Lausanne

par

Julien DELOBEL

Master en protéomique de l'Université des Sciences et Technologies de Lille 1

Jury

Prof. Margot Thome Miazza, Président
Prof. Jean-Daniel Tissot, Directeur de thèse
Prof. Michel Duchosal, expert
Prof. Nicolas Fasel, expert
Prof. Beat Riederer, expert
Dr. Philippe Tacchini, expert

Lausanne 2014

UNIL | Université de Lausanne
Faculté de biologie
et de médecine

Ecole Doctorale

Doctorat ès sciences de la vie

Imprimatur

Vu le rapport présenté par le jury d'examen, composé de

Président	Madame	Prof. Margot **Thome Miazza**
Directeur de thèse	Monsieur	Prof. Jean-Daniel **Tissot**
Experts	Monsieur	Prof. Nicolas **Fasel**
	Monsieur	Prof. Michel **Duchosal**
	Monsieur	Prof. Beat **Riederer**
	Monsieur	Dr Philippe **Tacchini**

le Conseil de Faculté autorise l'impression de la thèse de

Monsieur Julien Delobel

Master of Science de l'Université des Sciences et Technologies de Lille, France

intitulée

PROTEOMICS OF OXIDATIVE LESIONS OCURRING DURING TRANSFUSION-PURPOSED STORAGE OF ERYTHROCYTE CONCENTRATES

Lausanne, le 11 décembre 2014

pour La Doyenne
de la Faculté de Biologie et de Médecine

Thome-Miazza

Prof. Margot Thome Miazza

Abstract

Erythrocyte concentrates (ECs) are the major labile blood product being transfused worldwide, aiming at curing anemia of diverse origins. In Switzerland, ECs are stored at 4 °C up to 42 days in saline-adenine-glucose-mannitol (SAGM). Such storage induces cellular lesions, altering red blood cells (RBCs) metabolism, protein content and rheological properties. A hot debate exists regarding the impact of the storage lesions, thus the age of ECs on transfusion-related clinical adverse outcomes. Several studies tend to show that poorer outcomes occur in patients receiving older blood products. However, no clear association was demonstrated up to date. While metabolism and early rheological changes are reversible through transfusion of the blood units, oxidized proteins cannot be repaired, and it is likely such irreversible damages would affect the quality of the blood product and the efficiency of the transfusion. *In vivo*, RBCs are constantly exposed to oxygen fluxes, and are thus well equipped to deal with oxidative challenges. Moreover, functional 20S proteasome complexes allow for recognition and proteolysis of fairly oxidized protein, and some proteins can be eliminated from RBCs by the release of microvesicles.

The present PhD thesis is involved in a global research project which goal is to characterize the effect of processing and storage on the quality of ECs. Assessing protein oxidative damages during RBC storage is of major importance to understand the mechanisms of aging of stored RBCs. To this purpose, redox proteomic-based investigations were conducted here. In a first part, cysteine oxidation and protein carbonylation were addressed via 2D-DIGE and derivatization-driven immunodetection approaches, respectively. Then, the oxidized sub-proteomes were characterized through LC-MS/MS identification of proteins in spots of interest (cysteine oxidation) or affinity-purified carbonylated proteins. Gene ontology annotation allowed classifying targets of oxidation according to their molecular functions. In a third part, the P20S activity was evaluated throughout the storage period of ECs, and its susceptibility to highly oxidized environment was investigated. The potential defensive role of microvesiculation was also addressed through the quantification of eliminated carbonylated proteins.

We highlighted distinct protein groups differentially affected by cysteine oxidation, either reversibly or irreversibly. In addition, soluble extracts showed a decrease in carbonylation at the beginning of the storage and membrane extracts revealed increasing carbonylation after 4 weeks of storage. Engaged molecular functions revealed that antioxidant (AO) are rather reversibly oxidized at their cysteine residue(s), but are irreversibly oxidized through carbonylation. In the meantime, the 20S proteasome activity is decreased by around 40 % at the end of the storage period. Incubation of fresh RBCs extracts with exogenous oxidized proteins showed a dose-dependent and protein-dependent inhibitory effect. Finally, we proved that the release of microvesicles allows the elimination of increasing quantities of carbonylated proteins.

Taken together, these results revealed an oxidative pathway model of RBCs storage, on which further investigation towards improved storage conditions will be based.

Résumé

Les concentrés érythrocytaires (CE) sont le produit sanguin le plus délivré au monde, permettant de traiter différentes formes d'anémies. En Suisse, les CE sont stocké à 4 °C pendant 42 jours dans une solution saline d'adénine, glucose et mannitol (SAGM). Une telle conservation induit des lésions de stockage qui altèrent le métabolisme, les protéines et les propriétés rhéologique du globule rouge (GR). Un débat important concerne l'impact du temps de stockage des CE sur les risques de réaction transfusionnelles, certaines études tentant de démontrer que des transfusions de sang vieux réduiraient l'espérance de vie des patients. Cependant, aucune association concrète n'a été prouvée à ce jour. Alors que les modifications du métabolisme et changement précoces des propriétés rhéologiques sont réversibles suite à la transfusion du CE, les protéines oxydées ne peuvent être réparées, et il est probable que de telles lésions affectent la qualité et l'efficacité des produits sanguins. *In vivo*, les GR sont constamment exposés à l'oxygène, et sont donc bien équipés pour résister aux lésions oxydatives. De plus, les complexes fonctionnels de proteasome 20S reconnaissent et dégradent les protéines modérément oxydées, et certaines protéines peuvent être éliminées par les microparticules.

Cette thèse de doctorat est imbriquée dans un projet de recherche global ayant pour objectif la caractérisation des effets de la préparation et du stockage sur la qualité des GR. Evaluer les dommages oxydatifs du GR pendant le stockage est primordial pour comprendre les mécanismes de vieillissement des produits sanguin. Dans ce but, des recherches orientées redoxomique ont été conduites. Dans une première partie, l'oxydation des cystéines et la carbonylation des protéines sont évaluées par électrophorèse bidimensionnelle différentielle et par immunodétection de protéines dérivatisées. Ensuite, les protéines d'intérêt ainsi que les protéines carbonylées, purifiées par affinité, sont identifiées par spectrométrie de masse en tandem. Les protéines cibles de l'oxydation sont classées selon leur fonction moléculaire. Dans une troisième partie, l'activité protéolytique du protéasome 20S est suivie durant la période de stockage. L'impact du stress oxydant sur cette activité a été évalué en utilisant des protéines exogènes oxydées *in vitro*. Le potentiel rôle défensif de la microvesiculation a également été

étudié par la quantification des protéines carbonylées éliminées.

Dans ce travail, nous avons observé que différents groupes de protéines sont affectés par l'oxydation réversible ou irréversible de leurs cystéines. De plus, une diminution de la carbonylation en début de stockage dans les extraits solubles et une augmentation de la carbonylation après 4 semaines dans les extraits membranaires ont été montrées. Les fonctions moléculaires engagées par les protéines altérées montrent que les défenses antioxydantes sont oxydées de façon réversible sur leurs résidus cystéines, mais sont également irréversiblement carbonylées. Pendant ce temps, l'activité protéolytique du protéasome 20S décroit de 40 % en fin de stockage. L'incubation d'extraits de GR en début de stockage avec des protéines oxydées exogènes montre un effet inhibiteur « dose-dépendant » et « protéine-dépendant ». Enfin, les microvésicules s'avèrent éliminer des quantités croissantes de protéines carbonylées.

La synthèse de ces résultats permet de modéliser une voie oxydative du stockage des GRs, à partir de laquelle de futures recherches seront menées avec pour but l'amélioration des conditions de stockage.

Acknowledgements

First I would like to thank my thesis director, Prof. Jean-Daniel Tissot, for having given me the opportunity to conduct my PhD thesis within the Service Régional Vaudois de Transfusion Sanguine. Thanks to him, the transfusion center of Lausanne is one of the few to conduct transfusion medicine research out of the clinic domain. His enthusiasm for research is really appreciable, and his remarks and advices were always stimulating.

Next I would like to express special thanks to Dr. Niels Lion, head of research and development, blood component processing and quality assurance. I remember his sympathy and warm welcome at our first meeting. He is an extremely skilled supervisor, and he made me feel very comfortable at the beginnings of my PhD, letting me quickly manage my research independently.

I am very grateful towards Dr. Michel Prudent, head of the research unit, who accompanied me on the project of red blood cells storage lesions. We were long just two of us working on RBCs, against the platelet invaders… it necessarily created strong links. I really admire his qualities as a researcher, and the strong impact he had on his arrival on the development of our research group. Additionally, he is a very interesting and pleasant person. Thanks a lot for the critical reading and proofreading work on this manuscript.

I would like to thank also the peoples from the research unit: Mr. David Crettaz for his remarks questioning all your results, but which are, most of time, scientifically relevant! Thank you also for your great movie culture and for the pun contests. Mr. Giona Sonego: thanks for joining me as a PhD student and for lending me your couch the few nights when we got drunk! Dr. Mélanie Abonnenc: thanks for bringing a feminine touch to this team which desperately needed one! You also brought new interesting research perspectives through your experience in molecular biology. I also thank each intern who came in the lab, because I learnt from them as much as they learnt from us, and because they are plainly associated with the dynamics of the lab.

From the logistic team, I address a big thank to Guillaume Riat, with who I had the opportunity to spare frequent good times talking about common interests, football, anime and manga,

trading card games, allowing to clear mind from science at some times. I also thank Mr. Xavier Flambert who showed me a good way to decompress, playing squash (sorry again for your back!).

I would like to thank Anita Riat for her kindness and her infectious laugh. A little bit far from my family, I found in her like a second mother, and I am very grateful for that. And for the sandwiches as well! I also thank Claudia Gomez and Arlette Jauslin from the unit of blood component preparation. Their kindness was very appreciable. Claudia, you read me like an open book when I was sad, but you had the courtesy not insisting, which I appreciated.

To all people from the SRTS VD with whom I interacted during my PhD thesis, I say thank you for having took part in my adventure in Switzerland.

I thank very much William Kenji Duque, a dear friend encountered in Switzerland, with who I spent many nights playing trading card games, discussing and watching TV series and lots of movies. Lately, you made me go back to childhood, teaching me plenty of magic card tricks. I will never forget my 27th birthday, a simple meal with your kind daughters and you, but it really warmed my heart.

My final thank goes to my family from the north of France. I apologize for not having often visiting you all during my Swiss trip, but you were all in my heart and in my mind. I address a special thank to my parents, for their support which gave me the opportunity to achieve long studies, and for their love.

Now it is time for me to address my very final thank to the one who share my life for more than 7 years now, Dr. Mélanie Hillion. Since you came in my life, I feel like I am a better man, because you gave me self-confidence. Thank you for that. You gave me the strength to get over recent difficult times, and to achieve the present adventure. Thank you for being you, and thank you for being the other part of us.

PART 1.

INTRODUCTION

Table of contents

List of illustrations

List of tables

The history of blood transfusion

First observations and attempts

The story of blood transfusion really began with the discovery of the mechanics of blood flow in the human body by the English physician William Harvey, described in the "*Exercitatio Anatomica de Motu Cordis et Sanguinis in Animalibus*" in 1628. From this highly important improvement in human physiology knowledge and the vision of blood as the "river of life", it became possible to consider blood transfer as the way to cure pathological states, ranging from febrile state to mental disorders. In 1656, experimenting the feasibility of conveying liquid poison into a dog bloodstream, the English architect and scientist Christopher Wren designed an injection device that will be used for the first trials of blood transfusion. First blood transfusions are imputed to Richard Lower, British physician, in 1665, performing both animal-to-animal and animal-to-human transfusions. The latter being published 4 years later, the first published animal-to-human blood transfusion was thus attributed to the French physician Jean-Baptiste Denis, successfully curing a 16-year-old boy suffering from fever, thanks to sheep blood in 1667. However, further unsuccessful attempts led the transfusion of blood being restricted to animal experiments and prohibited in humans in France by the 1675 edict of Châtelet. Meanwhile, the high quality of microscope lens fashioned by Antoni van Leeuwenhoek, a Dutch scientist precursor of cellular biology and microbiology, allowed him to observe, among others, animal and human blood in 1674, and to describe it as composed of red corpuscles swimming into a clear liquid, thanks to an innovative and incomparable microscope for its time (see Figure 1).

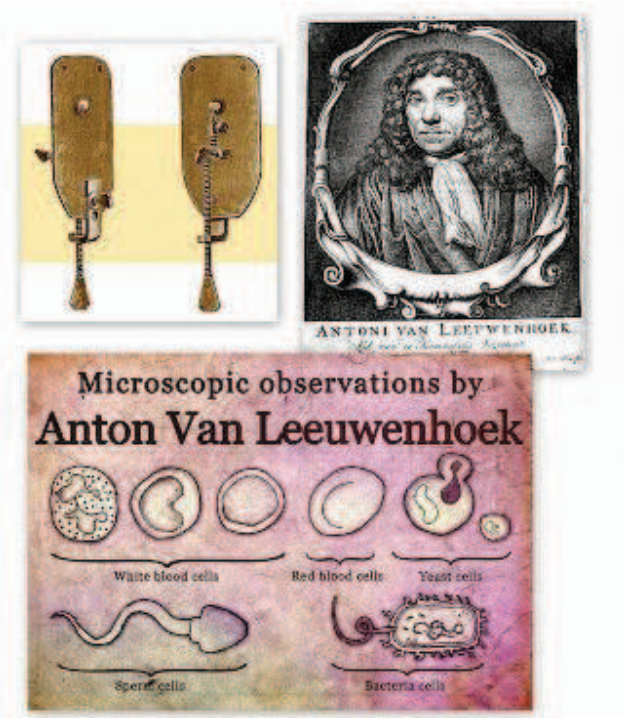


Figure 1: The microscope of Leeuwenhoek and some drawings of its observations.

Approximatively one and a half century later, the English obstetrician James Blundell realized the first successful human-to-human blood transfusion in 1829. He further documented several successful transfusions but also had some failures. Moreover, he contributed to the development of instruments for blood transfusion. In the second half of the XIXth century, two authors described the phenomenon of agglutination of the red cells of blood. In 1869, the German physician Adolf Creite, upon injection of serum from different mammals into rabbits, observed bloody urines free of intact red cells, as others previously noted while injecting whole blood. Creite concluded to chemical properties from some serum constituents, able to dissolve red cells. However, injection of protein-depleted serum did not induce blood-stained urines, attributing the dissolving role to serum proteins. Trying to support the role of such chemical agents in serum, other experiments in which sera from different mammals (except rabbit) were added to fresh rabbit blood allowed him to report for the first time the phenomenon of red cells agglutination, which he termed "accumulation". Half a decade later, in 1875, the German physiologist Leonard Landois extended Creite's experimentations, observing both the dissolution of transfused red cells and a "ball formation" of red cells in contact with sera from other animals. He noted differences in these activities, depending on the origins of blood/serum. When dissolution was not immediate, he was able to observe a change in red cells shape, in particular the formation of spikes and then a tendency to become spherical. Depending on the number of red cells added, he also remarked a tendency to accumulation rather than dissolution. He hypothesized that red cells were acquiring the ability to stick together while changing of shape, and that the dissolution capacity was due to an unknown ratio of the serum constituent parts. These observations from Creite and Landois led the community to progressively abandon animal-to-human transfusion because of the systematic lethal agglutination phenomenon. In the early 1900's, Karl Landsteiner, an Austrian biologist and physician, processed to interactions between blood serum and red corpuscles of different human individuals. In a first study (1900), he footnoted that

"*the sera of healthy individuals […] have an agglutinating effect […] on human red cells from different individuals*"[1].

[1] The translations from original German texts were reproduced from ref [1].

He thus associated agglutinogens to red cells and agglutinins to blood serum, but had doubts regarding a hypothetical disease-origin of serum agglutinins, as thought by others. One year later, he discovered the existence of 3 groups of blood, describing that

"*In many cases sera of group A react with red cells of another group, B, but not with group A; on the other hand, A red cells are affected in the same way by serum B. The sera of the third group (C) agglutinates red cells A and B, but C red cells are not affected by sera from A and B. Naturally the red cells must be considered insensitive to the agglutinins which are present in the same serum*"[1(page 5)]

Human-to-human blood incompatibilities were thus demonstrated by these studies which can be considered as the premises of the discovery of blood groups and transfusion compatibility rules, Landsteiner suggesting the existence of 2 agglutinins as an explanation of the 3 groups he observed. In 1902, Decastrello and Sturli, Austrian physicians, discovered the fourth blood group. In particular, the publication of the American physician and hematologist Reuben Ottenberg in 1911 [2] describes very well the isoagglutination issues from each possible combination of red cells and serum, as shown in Figure 2.

Interagglutination Reactions of Ten Individuals.

			Sera.									
Groups.			I				II	III				IV
			1	2	3	4	8	6	7	9	10	5
Cells	I	1	−	−	−	−	−	−	−	−	−	−
		2	−	−	−	−	−	−	−	−	−	−
		3	−	−	−	−	−	−	−	−	−	−
		4	−	−	−	−	−	−	−	−	−	−
	II	8	+	+	+	+	−	+	+	+	+	−
	III	6	+	+	+	+	−	−	−	−	−	−
		7	+	+	+	+	+	−	−	−	−	−
		9	+	+	+	+	+	−	−	−	−	−
		10	+	+	+	+	+	−	−	−	−	−
	IV	5	+	+	+	+	+	+	+	−	?	−

Figure 2: Discovery of the 4 blood groups. Table of cross-reactivity between serum agglutinins and red blood cells from the 4 discovered blood groups, as reported by Ottenberg in 1911. Reproduced from ref [2].

World Wars as the battlefields of developments and experimentations

The next steps towards current blood transfusion are related with World War I and the need to dissociate the blood donor from the recipient. Blood transfusion was a surgical act involving the dissection of veins or arteries from the donor and the recipient. The Carrel's suture and the Crile's cannula methods were the two techniques commonly used for end-to-end anastomosis of blood vessels (see Figure 3). Until then, transfusions were thus performed arm-to-arm, which was problematic in war times, meaning that donors of blood had to be present next to patients. In the early 1890's, citrate was shown to interfere with the clotting process [3] through calcium binding [4]. Wright even declared that citrated blood appeared more suitable for transfusion [5]. However, blood clotting was then a minor issue in comparison with the not yet understood group incompatibility-related agglutination. Moreover, sodium citrate was considered highly toxic thus unsuitable for human transfusion.

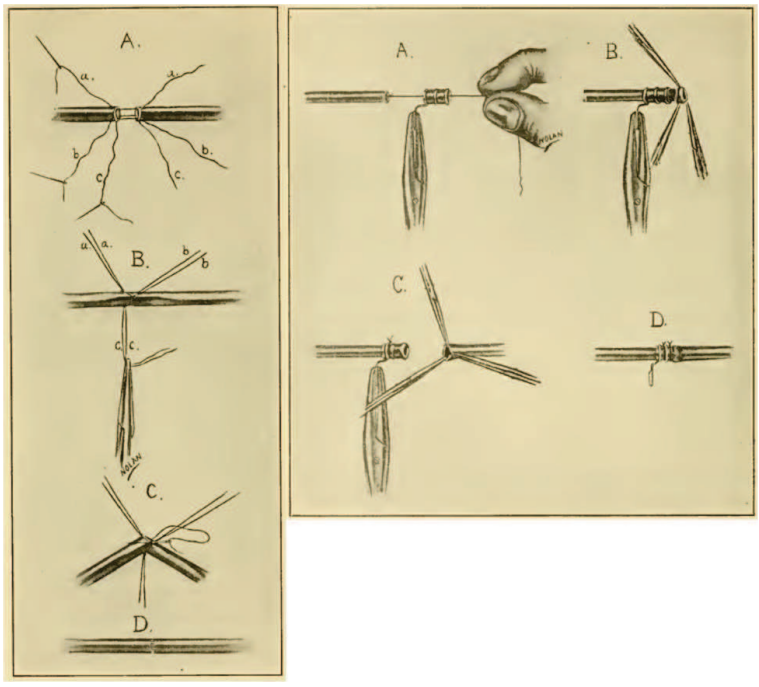


Figure 3: Schemes of stages of blood vessels anastomosis as performed by the end of the XIXth century. Left panel: Carrel's suture method; right panel: Crile's cannula method. Schemes were reproduced from ref [6].

Technical development of transfusion devices allowed puncturing blood from the donor and further injecting it in the recipient, but blood clotting often prevent success of such approaches. Blood clotting thus became a main issue in transfusion, and sodium citrate was reconsidered in 1914 by Albert Hustin, a Belgian physician, to prevent blood from coagulation. Independently, Hustin, Agote and Lewisohn performed the first transfusions of citrated blood to human in 1914-1915. This discovery allowing the spatial dissociation of recipient from the blood donor will however not be used until the end of the war. The first direct blood transfusion (arm-to-arm) of World War I was performed by the French physician Émile Jeanbrau in 1914. This transfusion was luckily successful though bloods of donor and recipient were not cross-matched to ensure their group compatibility, as suggested by Hektoen in 1907. And so were done most transfusions during the war. Indeed, the watchword was to take the risk of blood incompatibility issues rather than watching people dying from massive hemorrhage.

If sodium citrate was proven to keep blood liquid up to a few days, it did not make blood uncoagulable, and blood storage was not really feasible. The first success in erythrocyte conservation was achieved in 1916 by Rous and Turner who were able to store rabbit red blood cells in ice box for up to 4 weeks in a solution composed of sodium citrate and glucose, with reasonably low hemolysis [7, 8]. However, the transfusion of such stored RBCs back to rabbit was shown efficient only with cells stored for up to 2 weeks [8], which already highlighted the problem of storage-related aging of RBCs (see the section RBCs storage lesions, page 24). Thanks to this method, Oswald Robertson reported in 1918 the efficient storage, transport and transfusion of 22 “units” of human RBCs stored for up to 26 days, during rush periods at casualty clearing stations [9]. What Robertson described here can be considered as the world’s first blood bank. He also developed a specific bottle equipped with a negative or positive pressure pump device for blood collection in citrate solution or infusion of the citrated blood to the recipient (see Figure 4) [10].

World War I, through its duration and the number of involved countries, is considered as the theater of high developments and experimentations in blood transfusion. From isolated attempts to rejuvenate life, blood transfusion became a routine medical care for soldiers suffering from hemorrhage. After W.W.I, the main developments in transfusion consisted in

one hand in the implementation of developed techniques into general practice, and in the other hand in the identification and organization of potential donors for peace times.

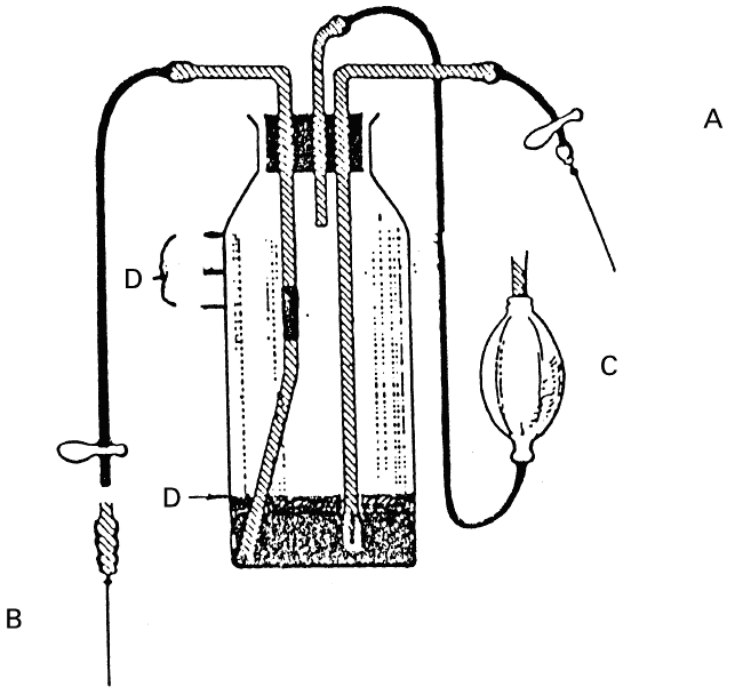


Figure 4: Robertson's bottle for citrated blood transfusion. Elements A and B are the puncture line for blood collection and the transfusion line to the recipient, respectively. The pumping device (C) allows applying negative or positive pressure to the bottle content for either collection or transfusion. Marks (D) indicate the volume of citrate solution (lower mark) and different volumes of blood collection (3 upper marks). The drawing is a reproduction from ref [10].

After war, technical development for transfusion was limited. Direct and indirect transfusion co-existed. Arm-to-arm transfusion was improved regarding the control of transfused blood volumes thanks the apparatus of Tzanck (1925) or the pump of Bakey (1935) for instance. Existing devices for indirect transfusion (for instance syringe, paraffin-coated tube or temporary storage bottle) were still in use in the 1930's, the real improvement being the effective use of sodium citrate. What especially marked the modern transfusion field between wars is the fact that this medical treatment was applied for routine care. From blood donation between soldiers, one had to begin recruiting donors from the civil population, calling for people solidarity (see Box 1).

Box 1: The selfless nature of blood donation

Wars allowed a lot of technical and scientific development. The high solidarity existing between comrades in arms, to fight for liberty against their enemies, and against death, allowed the experimentation of blood transfusion. During the First World War, several records of people altruism and solidarity demonstrated the beauty of blood donation, the gift of self, and the recognition of transfused peoples towards their benefactors, as illustrated by the first direct arm-to-arm transfusion presented in Figure 5. After W.W.I, the main application for blood transfusion became women birth-related hemorrhages. It is really symbolic that modern transfusion, developed to prevent death of victims from such a man-ascribed tragedy, became the insurance of happiness and life. The motivation for blood donation then appeared not so evident in comparison with war times.

Figure 5: First direct transfusion during the World War I. The text in A is a clipping from the French newspaper "l'ouest-Éclair" published in 1914, relating the first direct blood transfusion of W.W.I. The picture in B was taken during the transfusion process, realized by anastomosis of an artery from the donor with a vein of the recipient, as schematized in C. Henri Legrain, the recipient (on the right in picture D) was very thankful to his new "blood-brother", Isidore Colas, the donor (on the left in picture D).

The donation of blood is not an insignificant act. Blood as long been considered as the symbol of vitality and it is not so easy to say oneself "well, I am healthy so I will give part of my vitality to help other people". However, humans are, by nature, selfless and humanistic. Thanks to the development of blood storage, a big wave of solidarity took place during W.W.II. In France, the first transfusion center at the hospital Saint-Antoine in Paris (Dr. Arnault Tzank, 1923) organized the collection of citrated blood from the rear to supply the battle front, the only watchword being "no one injured would die for lack of blood". This awareness campaign was a resounding success among the population.

Box 1: The selfless nature of blood donation (continued)

In most countries, the donation of blood is a voluntary non-remunerated act. In 2005, Misje *et al.* conducted a questionnaire-based study of motivational characteristics of active blood donors at the blood bank of Oslo, Norway [11]. The questionnaire investigated four factors of motivation, labeled "value" for altruistic and empathic reasons for volunteering, "social" for the normative influence of friends, family, or a social group, "esteem" for the better feeling about oneself and "understanding" for the positive experiences associated with volunteering.

The study demonstrated that long-term donors were mostly motivated by altruism and empathic reasons (*e.g.* "it's important to help other people", "I feel compassion towards the receivers of blood products" or "blood donation is a cause that is important to me"), whereas donors presenting a short donation record appeared to be more supportive of self-esteem motives (*e.g.* "donating blood makes me feel needed", "makes me feel important" or "makes me feel better about myself").

The spread of the blood transfusion practice required more blood donations, and a better organization. Thus, the 1920's were witnesses of the creation of the first centers for blood transfusion, followed in the late 1930's by the establishment of blood banks. Spain gave birth in 1936 to the world's first blood bank during its civil war, rapidly followed by the United States of America as well as other European countries, coinciding with the beginning of World War II. Meanwhile, in 1940, Karl Landsteiner, Nobel Prize of physiology-medicine in 1930 for his discovery on human blood groups, highlighted the existence of the Rhesus factor with his colleague Alexander Wiener. In 1939, Levine reported a case of group O patient of which serum presented iso-agglutination (intragroup erythrocyte agglutination) with 80 % of tested group O bloods [12]. One year later, Landsteiner and Wiener showed that rabbit serum immunized with Rhesus monkeys (Macaca mulatta) blood was reactive to around 85 % of Caucasian humans' blood, in an ABO blood group-independent manner [13]. Landsteiner further confirmed that the majority of ABO-compatible transfusion reactions were caused by this antigen [14]. Since then, the process of blood transfusion (without regarding transfusion-related disease transmission) became a really secured procedure for recipients. The year 1940 also corresponds to the beginnings of plasma fractionation into its different protein constituents. In particular, Edwin Cohn brought the bases of plasma fractionation with 2 papers introducing the separation of equine or bovine serum upon equilibration across membranes using ammonium sulfate or ethanol dilutions of controlled pH, ionic strength, and temperature [15, 16]. The second paper also presented a detailed procedure to obtain 50 g of albumin from 2 L of plasma [16]. Cohn's method allowed producing therapeutic dried albumin units successfully used during W.W. II.

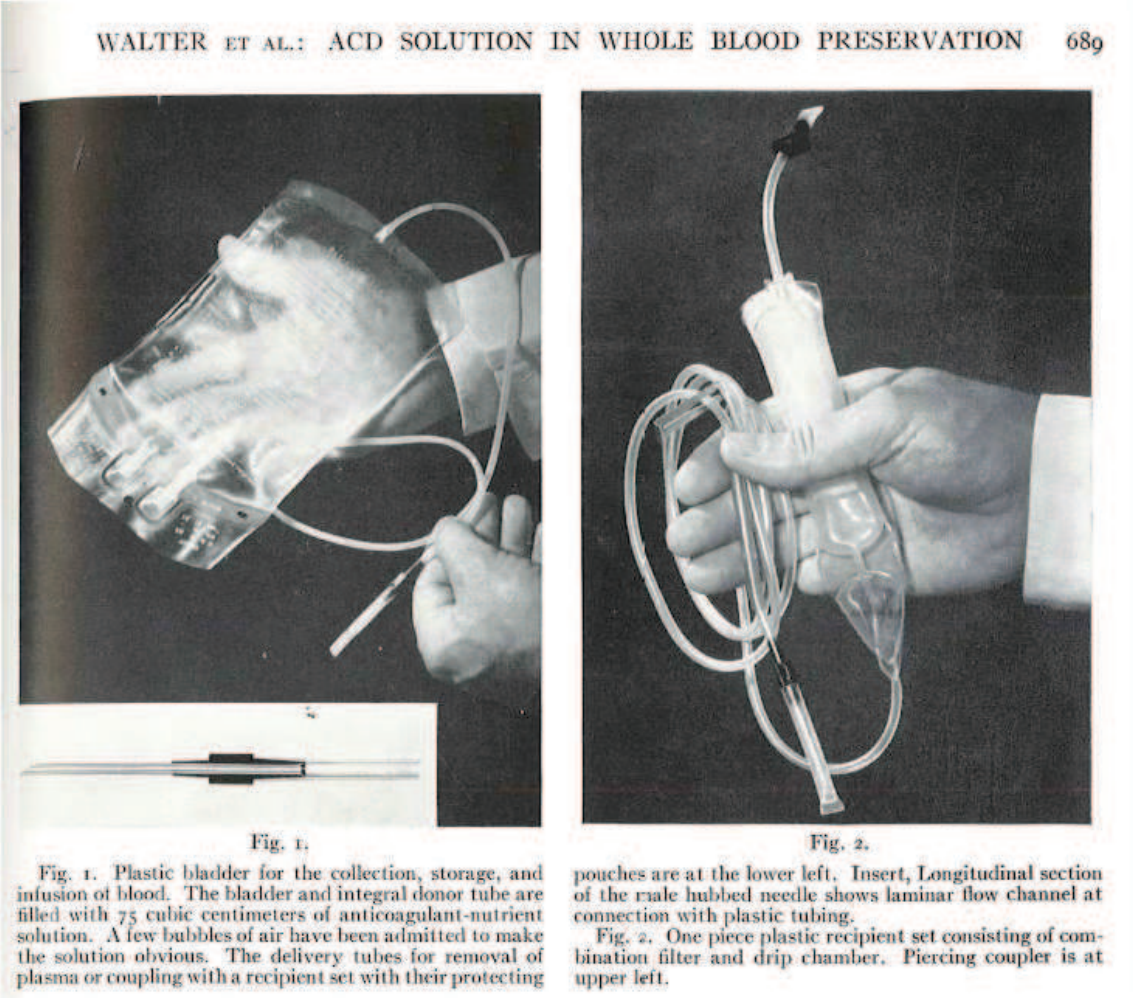
In 1943, a great advancement occurred for blood preservation. In order to avoid formation of caramel while autoclaving glucose-containing anticoagulant preservative solution due to its alkaline pH, Loutit, Mollison and Young tried counterbalancing less sodium citrate by addition of citric acid [17]. They screened blood storage at 3-7 °C during 4 weeks in preservative solutions presenting different sodium citrate/citric acid ratios, and observed degree of caramelisation and loss of glucose after autoclaving as well as end-storage hemolysis. Satisfactory solutions were further analyzed for post-transfusion *in vivo* survival and several biochemical parameters such as spontaneous hemolysis, osmotic fragility, pH, glucose,

potassium and formation of methemoglobin in comparison to currently used preservative solutions (Rous-Turner and the MRC (English Medical Research Council) solutions). The conclusions of this study were that acidified citrate-glucose (ACD) preservative solutions are satisfactory for blood storage and are recommended because of (1) a better survival in recipients circulation, (2) the low caramelisation while autoclaving the whole solution (until then, the alternative was the separated autoclave of trisodium citrate and glucose solutions followed by an aseptically mixing), (3) the absence of adverse outcome during and post-transfusion of ACD-stored blood, and (4) the absence of significant increase in storage-related methemoglobin formation in comparison with currently used preservative solutions. ACD blood preservative solution, allowing blood storage for 3 weeks (based on the 24h post-transfusion survival) will stay in use until the introduction of phosphate by Gibson in 1957 [18], which increased blood storage up to 4 weeks, through better maintenance of 2,3-DPG levels [19].

The afterwar period: towards the modern transfusion medicine

The 1950's correspond to a critical storage-related technical development. Carl Walter and William Murphy presented in 1952 the utilization of closed system of plastic bags (see

Figure 6) for blood collection, preservation in ACD, and transfusion [20]. The advantages were clear regarding bacterial contamination (no contact with air), lighter weight, shock resistance, and easiness of storage in refrigerators, in comparison with glass bottles. In 1954, in a publication from the army medical service, Artz and coworkers reported the use of plastic bags for blood transfusion during the war of Korea [21]. Analyses of plasma potassium levels in such bags revealed a favorable comparison with blood storage in glass bottles. Additionally, more recent research highlighted the role of the plasticizer (diethylhexyl phthalate, DEHP), released in blood bags, interacting with RBCs membranes and limiting the loss of membranes through the microvesiculation release [22]. However, plastic bags were not welcome on battlefields. Indeed, the gravity-related blood infusion to the recipient did not convey because *"about 30 per cent of the blood given in a forward surgical hospital must be given under pressure"*. Even if plastic bags could be squeezed for forcing blood out, *"it was impractical to assign one corpsman to each bag when 8 or 10 pressure transfusions were being given simultaneously to various patients"* [21].



WALTER ET AL.: ACD SOLUTION IN WHOLE BLOOD PRESERVATION 689

Fig. 1.

Fig. 1. Plastic bladder for the collection, storage, and infusion of blood. The bladder and integral donor tube are filled with 75 cubic centimeters of anticoagulant-nutrient solution. A few bubbles of air have been admitted to make the solution obvious. The delivery tubes for removal of plasma or coupling with a recipient set with their protecting pouches are at the lower left. Insert, Longitudinal section of the male hubbed needle shows laminar flow channel at connection with plastic tubing.

Fig. 2.

Fig. 2. One piece plastic recipient set consisting of combination filter and drip chamber. Piercing coupler is at upper left.

Figure 6: Plastic bag system for collection, storage and infusion of whole blood (reproduced from ref [20]).

Next steps in the improvement of blood storage dealt with the introduction of adenine as a constituent of preservative solutions, as proposed in 1962 by Nakao *et al.* [23]. This implementation takes its sources from the demonstrations made by the same group, that adenine and inosine enabled the regeneration of ATP and the modification of shape of long-stored RBCs [24-26] (see Figure 7, panel A), and that ATP level directly impacts the *in vivo* viability of red cells [27] (see Figure 7, panel B). CPDA-1 and its modified version CPDA-2 were licensed in USA during the late 70's / early 80's, while transfusion medicine already moved to the use of packed RBCs concentrates. Sodium-Adenine-Glucose (SAG) was the first additive solution for storage of packed RBCs units [28], further modified by addition of mannitol,

becoming SAGM, for a reduced end-storage hemolysis [29]. Other additive solutions deriving from the original SAG one are used worldwide such as AS-1, AS-3 and AS-5 (USA and Canada) or MAP (Japan). The rules of the 75 % of survival in recipient 24 h post-transfusion and less than 1 % (sometime 0.8 %) of hemolysis allow the storage of erythrocyte concentrates (ECs) in SAGM or derivatives for up to 6 weeks at 4 °C.

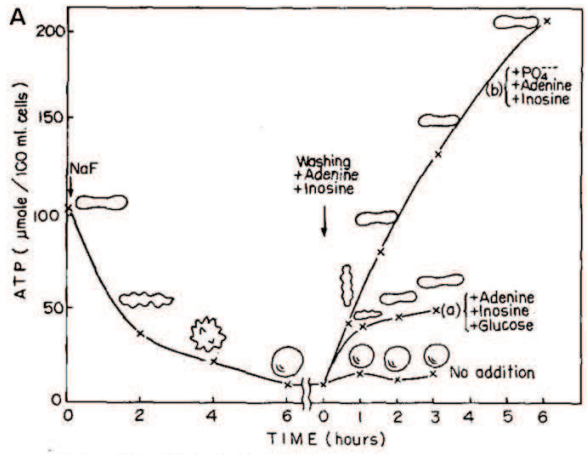


FIG. 3. Washed erythrocytes were incubated with NaF at 37°C for 6 hours and after washing out fluoride, reincubation was carried out with addition of inosine, adenine and glucose (a), and with addition of adenine, inosine and inorganic phosphate (b).

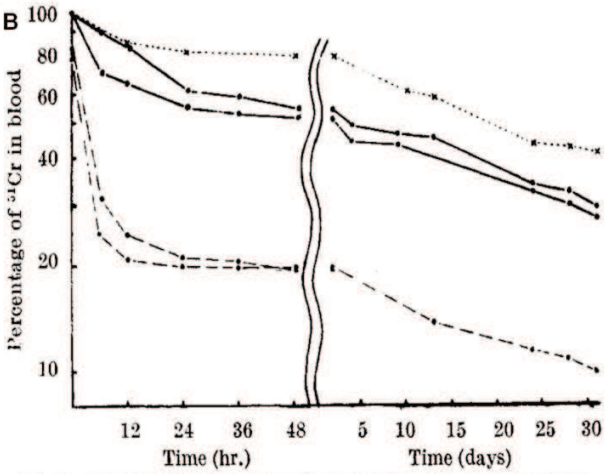


Fig. 1. Decay of radioactivity in circulating blood after transfusion of erythrocytes stored for eight weeks and labelled with radioactive chromate. Radioactivity of the blood 5 min. after the transfusion was represented as 100 per cent. ●—●, Erythrocytes stored in ACD and then incubated with inosine and adenine at 37° C. (specimen *A*); ×---×, erythrocytes stored in ACD containing inosine and adenine (specimen *B*); ●– –●, erythrocytes stored in ACD alone (specimen *C*)

Figure 7: Effects of adenine and inosine on ATP level, shape, and *in vivo* viability of stored RBCs. Panels A and B are reproductions from ref [26] and [27].

The last improvement of blood transfusion regarded the removal of leukocytes during the processing of ECs. Leucocyte cold lysis release damaging content for RBCs. The leukoreduction is performed either by removal of the buffy coat layer after whole blood centrifugation, and/or by leukofiltration. In addition to better maintainance of RBCs, leukocyte depletion prevents transmission of viruses from the donor to the recipient. More recently, a retrospective study also demonstrated decreased rates of febrile non hemolytic transfusion reactions (FNHTRs) in case of systematic prestorage leukodepletion [30].

Blood transfusion in Switzerland

The Swiss Red Cross and its transfusion service

Since the beginning of World War II, and more precisely October 9th 1939, The Swiss Red Cross (SRC) was asked to recruit blood donors among the civil population in order to establish a military blood transfusion service. At the end of this terrible conflict, the US Red Cross offered lots of dried plasma units to be distributed in Swiss hospitals, which led the SRC to set up a blood transfusion service intended for peace times. In particular, the Swiss federal decree of June 13th 1951 concerning the SRC (513.51) states that (1) SRC must provide help to the military health service in case of armed conflict, and (2) its main tasks are the voluntary humanitarian aid, the blood transfusion service for military and civilian needs, and the encouragement of nursing care. The blood transfusion service entrusted to SRC is now delegated to the SRC transfusion service (T-CH). T-CH is requested to conduct the national collection of blood, to maintain a national blood reserve, and to guaranty the respect of prescriptions, methods and materials in the regional blood services. Nowadays, 13 regional centers assume the blood supply in Switzerland through the collection, preparation and distribution of blood products to hospitals and physicians from their region. As a therapeutic product, blood – and its derivatives – collection, storage and utilization are controlled by the competent authority Swissmedic.

Current collection and storage of RBCs concentrates

As shown above, many efforts have been made to improve transfusion processes, from the collection to the infusion, through the storage of blood products. Labile blood products are nowadays transfused independently, since each of them can cure different pathological status. Indeed, RBCs units are transfused to cure anemia due to hemorrhage, medullar insufficiency, and hemoglobin or erythrocyte membrane synthesis anomalies. Platelet concentrates (PCs) and plasma units are transfused in case of hemorrhagic or hemostatic disorders, respectively. Additionally, storage of each component is optimized in accordance to its intrinsic properties, which leads to an overall optimal availability of all components. Such a case-dependent transfusion implies to manage each blood component independently. Blood products are

obtained either by processing whole blood donations or collecting them individually by an apheresis-driven donation of the needed component. This also implies to store each component individually, in conditions that are optimized to preserve their specific activities and functions. ECs are actually the main labile blood product to be transfused (75 to 80 % of delivered blood products in Switzerland during the past 5 years, see Figure 8). In Lausanne, all above-listed improvements led the transfusion medicine community to collect, process and store RBCs as follow. Whole blood donation (450 ± 50 mL drawn in 63 mL of CPD anticoagulant solution) is held at 22±2 °C overnight and is then centrifuged at 3500 *g* during 14 minutes to separate RBCs, plasma, and an intermediate buffy coat layer rich in platelets. RBCs and plasma are extracted from the original blood donation bag by a semi-automated pressure. Erythrocytes are then transferred into a SAGM-containing bag to a total volume of 275±75 mL and a hematocrit of 0.6±0.1. Then, a leukoreduction step is performed by filtration through an integrated filter. In these conditions, ECs can be stored at 4 °C up to 6 weeks. The duration of storage of RBCs units is a hot matter of debate. The community wonder if long-stored blood products are still of the required quality for the patient who need it. Several studies have been conducted, in different countries, on the use of blood products that are differently processed, and in different pathological cases, to investigate the impact that long-stored blood products could have for the recipient, in comparison with short-stored products.

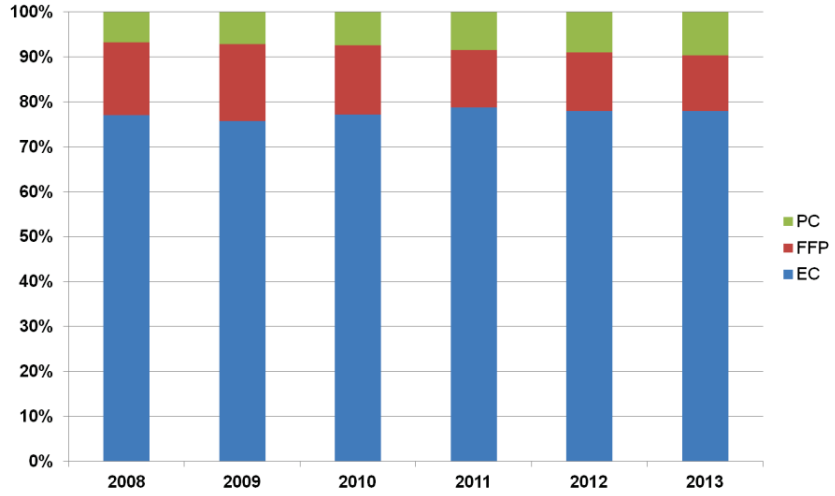


Figure 8: Repartition of labile blood products delivered in Switzerland from 2008. PC = platelet concentrate, FFP = fresh frozen plasma and EC = erythrocyte concentrate. Data published by Swissmedic in the 2013 hemovigilance report [31].

Transfusion of RBCs units in Lausanne

Depending on local blood management policies, the blood units' delivery may vary. In Lausanne, the priority of delivery is put to oldest (but not perished) available blood units of the required blood group, in order to avoid wasting blood products (rule of first-in, first-out). However, some exceptions are allowed, for instance in case of emergency, there is no time for determining the patient blood group and O group blood units are thus transfused. Another situation is the pediatric transfusion, where short-stored units are given because of the toxicity of high extracellular potassium levels to children. Statistics of transfused ECs blood groups at the university hospital of Lausanne (CHUV) are presented on Figure 9. Most of EC units are transfused during their 1st month of banking, as shown in panels A and B. In particular, averaging all blood groups, 90.4 % of RBCs units are transfused by the end of the 4th week of storage, with a peak of 39.4 % of transfused units during the 2nd week of storage. Blood groups O and A are the most transfused ones, as shown in Figure 9C. It is noteworthy that B and AB units (less transfused because more rare blood groups in the population) are mostly transfused during the 2nd half of the storage period, as shown in Figure 9D. In particular, the AB^+ blood units are highly transfused during the last week of storage, reaching nearly 60 % of AB^+ RBCs transfusion (see graphic A, light blue drawing).

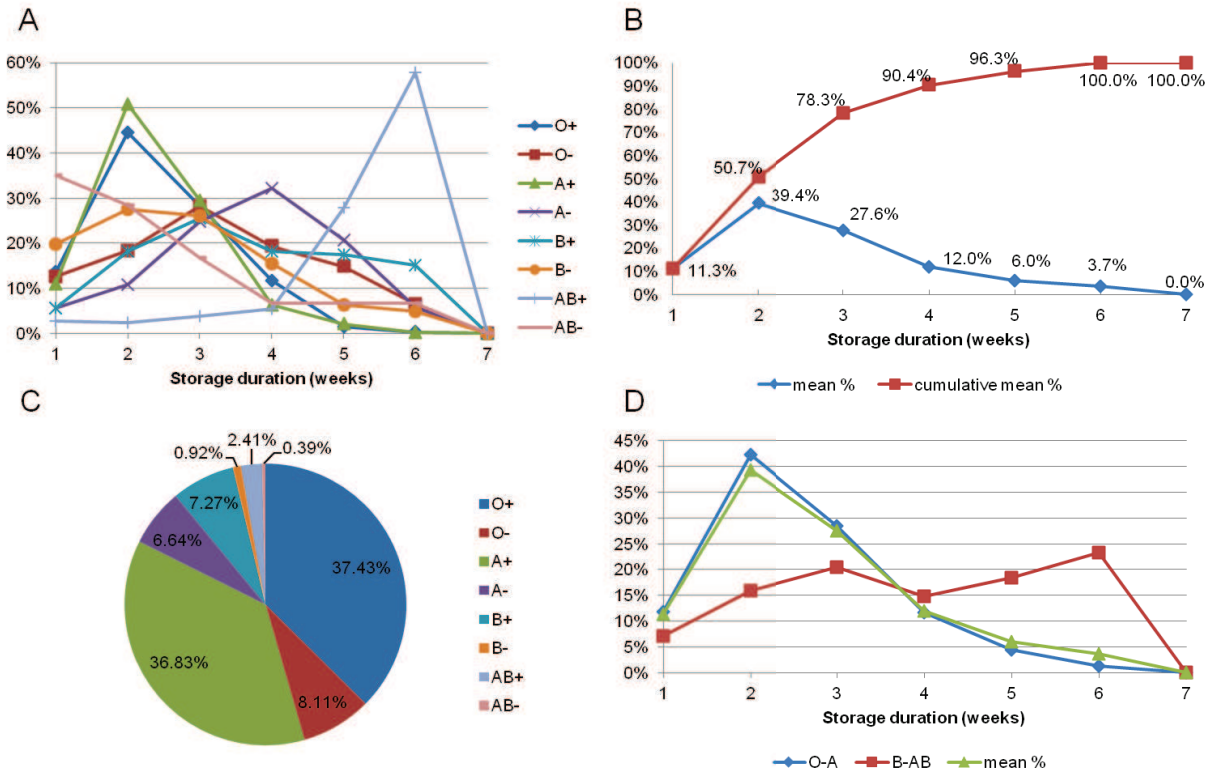


Figure 9: Statistics of transfused ECs blood groups and age of transfused ECs in Lausanne. These statistics are based on the transfused ECs at the CHUV during the period 2011-2012. For each blood group, the proportion of ECs being transfused during each week of the storage period is presented in A. Graph B shows the mean proportion of ECs transfused during each week as well as the mean cumulative proportion of ECs transfused throughout the storage period. The proportion of transfusion of each blood group appears in the piechart C. Graph D shows the comparison of the mean proportions of abundant blood groups (O and A) *versus* minority blood groups (B and AB) transfused during each week of the storage duration, as well as the mean proportions of whole EC units transfused. Data were adapted from the medicine master thesis of Marine Gossin [32].

Transfusion efficiency and clinical outcomes

Adverse events and outcomes of blood transfusion

Blood transfusion process can be accompanied by adverse events occurring during the process (transfusion reaction) or over a 24h post-transfusion delay (post-transfusion complications). The severity of these events can be very moderate or really severe. It is possible to classify the different transfusion adverse events, taking into account the pathogenesis (immune reaction?, infectious transmission?), the type of reaction (febrile or allergic) and the time from transfusion to development of the event (acute vs delayed). Most common events found in the literature are acute transfusion reaction (ATR), hemolytic transfusion reaction (HTR), incorrect blood component transfused (IBCT), multiple organ dysfunction syndrome (MODS), post-transfusion purpura (PTP), transfusion-associated circulatory overload (TACO), transfusion-associated dyspnea (TAD), transfusion-related acute lung injury (TRALI), transfusion-associated graft-versus-host disease (TA-GvHD) and transfusion-transmitted infection (TTI). For the patient, transfusion-related events can correspond to increased length of hospitalization, post-operative ventilation, deep venous thrombosis, respiratory syndrome, (multiple) organ failure, organ rejection, infectious complications, or death. In 1993, Chiu *et al.* published a poster abstract into which they presented data of a prospective study showing that blood transfusion for ICU (intensive care unit) patients was associated with the development of infections as well as MODS [33].

The relation between adverse events and the transfusion in critically ill patients has been investigated through a systematic review of the literature in 2008 by Marik and Corwin [34]. Data from 45 studies (approximately half retrospective and half prospective cohorts) allowed them to conclude to an association of RBCs transfusion with an increased morbidity and mortality in 93 % of the cases. Most studies seem to associate transfusion of blood to outweighed complication risks compared to benefits. However, several factors which are not systematically taken into account appear of high importance when performing such investigations. In particular, the age of the patient, the severity of illness requiring the transfusion of blood, the blood group and the number of blood units being transfused are

critical parameters. The transfusion of blood may appear at risk but adverse outcomes may in fact be due to a bad health context. In the same way, the more transfusion needed the more critical situation and the higher morbidity.

Age of blood products

The potential role of blood transfusion in adverse outcomes has been further investigated regarding the age of the transfused blood products, *i.e.* their duration of storage before use. An overview of the literature shows that the age of transfused blood was not taken into account regarding transfusion-related complications until late 1990's, though blood storage was questioned regarding the biochemical efficacy of transfusion (for instance oxygen delivery, see reference [35]). Purdy and coworkers reported for the first time in 1997 the association of storage duration with survival through a retrospective study on septic ICU patients (admission diagnosis) [36]. They did not found any significant difference in age, sex, absolute number of transfused ECs or length of stay at the ICU, but the mean age of ECs transfused to patients who survived was significantly lower than for non-survivors (17 days *vs.* 25 days of storage in CPDA-1, respectively). It was noteworthy that survivors received a high proportion (85 %) of ECs stored for less than 10 days and non-survivors received a high proportion (76 %) of ECs stored for more than 20 days. Blood groups were not taken into account. In another retrospective study, Vamvakas highlighted an association between wound infection or pneumonia and the storage duration of transfused RBCs in patients receiving a coronary artery bypass graft surgery [37]. Different complications were thus linked with age of blood in many disease contexts. The introduction of leukoreduction made several authors reporting a beneficial effect of the process on known adverse outcomes [38, 39]. It appeared that many post-transfusion complications were due to the immunomodulative effect attributed to residual leukocytes in RBCs units [40, 41]. Moreover, leukocyte depletion was recently shown to improve RBCs storage in terms of biochemical alteration they undergo during storage (this point will be tackled thereafter) [42].

Globally, many studies assessed the impact of age of RBCs on transfusion-related adverse clinical outcomes. Most of them impute complications to long-stored RBCs [43-49], with more

or less confidence due to the way they were conducted (number of studied cases; control conditions; data correction with respect to confounding patient-dependent parameters: age, sex, blood group, severity of illness, number of transfused blood units; construction of the study: retrospective *vs.* prospective, randomized or not, blinded or not, dichotomization approach?). In particular, the study conducted by Koch in 2008 on the role of aged RBCs (more than 14 days of storage) on cardiac surgery patient outcomes had a high impact on the transfusion medicine community (see Figure 10) [49]. Other studies also reported no effect of RBCs storage duration [50-54] and a few even concluded to worse adverse outcomes when fresher blood was transfused [50]. It also appeared that conclusions could be dependent of the number of transfused units [55]. Available literature presents very differently conducted studies, which makes it difficult to realize meaningful meta-analyses. A particular issue concerns geography. Indeed, processes of preparation of blood products are different in America and in Western European countries for instance. As mentioned by Van de Watering, characteristics of production and storage of ECs, such as donation type, overnight held at room temperature, leukocyte reduction method, preparation of RBC component, type of bags and additive solution used, should be mentioned to facilitate meta-analyses [56].

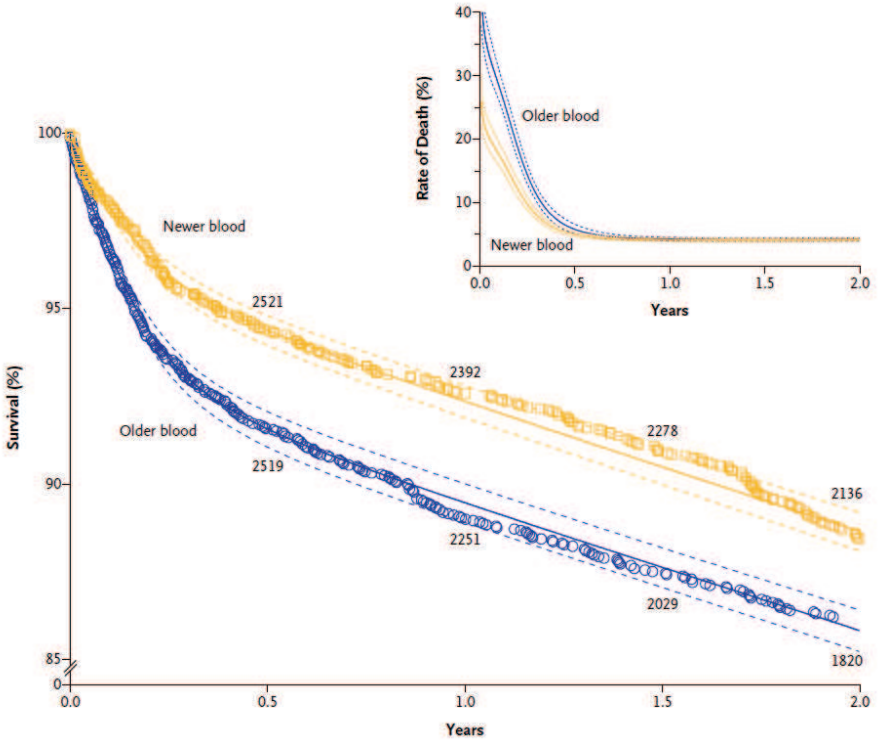


Figure 10: Kaplan-Meier estimates of survival and death post-cardiac surgery in function of age of transfused RBCs. The figure is reproduced from the controversial study of Koch [49].

RBCs storage lesions

If the clinical relevance of “old-ECs” transfusion on recipient adverse outcomes is still uncertain, there is no doubt about changes at the cellular and molecular levels which increase together with the storage duration. Three kinds of lesions occurring at the cellular and intracellular levels during RBCs cold-storage are now well described. First, erythrocytes undergo biochemical lesions, altering their metabolism. In RBCs occurs the anaerobic glycolysis, exclusively cytoplasmic, due to the absence of mitochondria. As a consequence, energy metabolism through glucose consumption leads to the production of lactate, which has been shown to accumulate in the RBCs storage solution [57], inducing its acidification [58]. The metabolism-related pH fall retro-inhibits the glycolysis rate. Level of 2,3-diphosphoglycerate (2,3-DPG) is affected by the pH, through the activities of diphosphoglycerate mutase and phosphatase. In stored RBCs, 2,3-DPG was thus shown to be depleted after 2 weeks [59]. Low level of 2,3-DPG induces an increased affinity of hemoglobin for oxygen, inhibiting its release and thus altering the oxygenation capacity of stored RBCs. However, 2,3-DPG depletion appears to recover in a few hours post-transfusion. The cold storage of RBCs induces inactivation of membrane ionic pumps, as shown by Na^+ uptake and K^+ loss throughout the storage period [59]. Low temperature also slows down metabolic enzymes activities, inducing a progressive decrease of ATP level [59]. D’Alessandro *et al.* also highlighted an activation of the oxidative phase of the pentose phosphate pathway, through accumulation of the metabolic intermediates NADPH and 6-phosphogluconate [57]. NADPH is needed for the reduction of oxidized glutathione (GSSG), recycling reduced GSH to fight against accumulating reactive oxygen species (ROS). However, GSH level was shown to continuously decrease over the RBCs storage [57]. There is a contradiction in the literature about GSSG level, sometimes increasing [57] or decreasing when no concomitant activation of the PPP is observed [60].

Constantly exposed to oxygen fluxes, RBCs also have to deal with oxidative stress. Erythrocytes are known to contribute to the maintenance of circulatory antioxidant levels, particularly through recycling oxidized forms of plasma ascorbic acid [61], and contributing to the extracellular pool of glutathione (GSH) [62]. However, RBCs can also act as ROS generators,

despite their characteristic absence of mitochondria. Indeed, the dissociation of oxyhemoglobin to deoxyhemoglobin through oxygen release can lead to iron electron capture by oxygen. Methemoglobin presents a ferric (Fe^{3+}) iron state and cannot bind oxygen, but can be converted back to Fe^{2+} normal hemoglobin (Hb) thanks to the enzyme methemoglobin reductase. This Hb autoxidation, occurring at a 3 % rate, leads to the formation of the superoxide radical $O_2^{\bullet-}$ [63], further producing highly reactive hydroxyl radical $HO^{\bullet}$ through the iron-catalyzed Haber-Weiss reaction [64]. Such radicals are extremely reactive and thus possess a very short half-life, estimated 10^{-9} seconds [65]. Hence, they can unspecifically affect all types of macromolecules of their near environment. In particular, lipid peroxidation and protein amino acids modifications or protein backbone breaks have been characterized as RBCs storage lesions [66, 67]. A particular interest is put on the oxidative lesions, since such damages cannot be reversed through the transfusion process, contrarily to biochemical modifications. Since erythrocytes are unable to perform protein synthesis, no protein turnover is possible. Damaged proteins accumulate until being degraded or eliminated out of the cell. Redox proteomic studies performed on stored RBCs have shown increasing hallmark of oxidative stress throughout the storage period. More precisely, protein carbonylation investigations bring Lion's [68], Papassideri's [69, 70] and Zolla's [57] groups to conclude an increase in carbonylated proteins content, associated to the RBC membrane and cytoskeleton for the two formers, or to entire erythrocytes for the latter. Zolla and colleagues also demonstrated that protein carbonylation was due to an increasing oxidative stress concomitant with an over-activation of the oxidative phase of the pentose phosphate pathway, recruited to fight against ROS. Protein oxidation can also be investigated through their cysteine redox status, but such approach has not been described in the transfusion field yet. As a consequence of oxidation, hemoglobin has been shown to crosslink as hemichromes that covalently bind the cytoplasmic domain of the major integral glycoprotein of erythrocyte membrane, Band 3, inducing its clustering [71-73]. The induced conformational change is known to create a neo-antigen site at the erythrocyte surface [74, 75], allowing *in vivo* recognition by naturally occurring auto-antibodies [76-80], leading to RBCs clearance from circulation through activation of the complement [81] and phagocytosis by Kupffer liver cells [82, 83]. This is known as the Band 3 clustering model.

Hemichromes are also capable of autoxidation, producing ROS in the membrane and cytoskeletal environment. Protein carbonylation is a hallmark of oxidative modifications, and it has been shown to increase in cytoskeletal protein population during RBCs storage [70]. Such alteration of the cytoskeleton involves a third type of storage lesions that are biomechanical lesions. Indeed, rheological properties of RBCs appear to be altered all along the storage period, directly impacting the *in vivo* capacities of transfused RBCs. They become more rigid and are thus less susceptible to flow through the capillary micro-circulation, diminishing their tissue oxygenation power. The most remarkable one is the modification of the shape, evolving from a normal discoid shape to spherocytes, with intermediate echinocyte and sphero-echinocyte shapes [84] (see Figure 11 for illustration). Along with this shape change, RBCs are known to release increasing amounts of microvesicles (MVs) [85], and present a loss of deformability [86], a tendency to aggregate [84], an enhanced adherence to endothelial cells [87] and an increasing susceptibility to hemolysis [88]. As for the microvesiculation, this phenomenon is believed to be a protective mechanism [89] since altered Band 3 (initiating RBCs clearance) is found enriched in MVs [90].

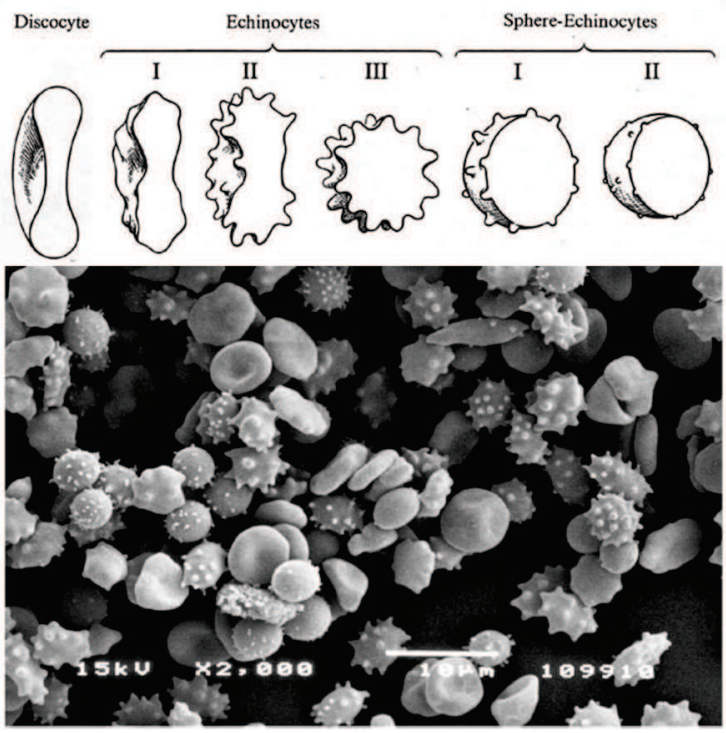


Figure 11: RBCs shape changes during storage. Electron micrograph is from a 42-day sample. Adapted from [57].

Ongoing research to improve RBCs-transfusion efficacy and safety

In a global manner, the doubt concerning transfusion-related adverse outcomes due to aged blood products is not really an issue because most ECs appears to be transfused during the first half of storage. The dichotomization of young and old blood products was shown to be not helpful for assessing an age-related issue of stored RBCs transfusion, and authors of such studies are not in accordance with each other regarding the limit of time qualifying stored blood as young or old. If clinical studies allow one day characterizing such a limit, time will be to change additive solutions, storage conditions, pre-transfusion practices or even hospital policies to better preserve blood products, to deliver safer products or patient-specific products, or to limit the needs for transfusion. Efforts are being conducted prospectively in this sense. Several groups are already developing new solutions for better RBCs preservation as well as special storage conditions such as anaerobic storage, and original pre-transfusion practices such as washing end-stored RBCs.

Additive solutions

As seen before, the storage of RBCs products constituted a big improvement for blood transfusion. In addition, the development of solutions allowing the current preservation of erythrocytes for 6 weeks has been a long and slow process. Current worldwide used additive solutions are approved by local drug administrations. However, the storage of RBCs is not optimal regarding the 120-day lifespan of red cells *in vivo*. Research is conducted in order to improve the existing additive solutions, either to improve the quality of stored RBCs or to prolong their storage duration. In particular the group of Hess published 4 papers in 4 years in the early 2000's, introducing experimental storage solutions (EAS) allowing the effective preservation of RBCs for 9 weeks in EAS-61 [91], 10 weeks in EAS-64 [92], 11 weeks in EAS-67 [93] and 12 weeks in EAS-76 [94]. In these publications, Hess and colleagues showed progressive changes in AS-1-derived EAS to prolong more and more the storage duration of RBCs while keeping a hemolysis below 1 % and an *in vivo* 24-hour recovery higher than 75 %. Table 1 shows the compositions of currently approved AS as well as other EAS investigated by Hess *et al.* for RBCs banking. In particular, lower salt concentration, higher adenine content

(higher AS volume) and a more alkaline AS pH seem to let RBCs ATP and 2,3-DPG concentrations longer preserved, in comparison with the mostly used SAGM and AS-1/3/5 solutions. The main drawback of designed EAS is the alkaline pH which favors dextrose caramelization during the heat sterilization process. Components should thus be autoclaved separately then mixed aseptically. Current and new additive solutions were thus shown to allow the RBCs storage more or less longer, regarding their stability in blood bags and *in vivo* (hemolysis and survival). However, regarding all storage lesions described so far, one can wonder if other aging markers should not be taken into account for determining an optimal storage period.

Table 1: Composition of additive solutions for RBCs storage

Constituents (mM)	SAGM	AS-1	AS-3	AS-5	MAP	PAGGSM	EAS-61	EAS-64	EAS-67	EAS-76
Adenine	1.25	2	2	2.2	1.5	1.4	2	2	2	2
Dextrose	45	111	55	45	40	47	110	50	50	50
Mannitol	30	41	-	45.5	80	55	55	20	20	30
NaCl	150	154	70	150	85	72	26	75	75	45
Na_2HPO_4	-	-	-	-	-	16	12	9	9	9
NaH_2PO_4	-	-	23	-	6	8	-	-	-	-
$NaHCO_3$	-	-	-	-	-	-	-	-	30	30
Citric acid	-	-	2	-	1	-	-	-	-	-
Na-citrate	-	-	23	-	5	-	-	-	-	-
Guanosine	-	-	-	-	-	1.4	-	-	-	-
pH	5.7	5.5	5.8	5.5	5.7	5.7	8.2	8.5	8.4	8.4
Volume (mL)	100	110	110	100	92	100	200	300	300	300

Rejuvenation

Another possibility for the transfusion of ECs of better quality, instead of developing more efficient additive solutions, can consist in the rejuvenation of stored red cells before transfusion. Rejuvesol™ is a solution composed of 100 mM Na-pyruvate, 99.9 mM inosine, 5 mM adenine, 70.4 mM Na_2HPO_4 and 29 mM NaH_2PO_4, allowing to replenish depleted ATP and 2,3-DPG levels by reactivating RBCs metabolism through a 1-hour incubation at 37 °C. Afterwards, rejuvenated cells should be washed to remove excess of inosine, potentially toxic for the recipient. Meyer *et al.* showed efficient ATP and 2,3-DPG increase through this process on ECs samples from AS-1, AS-3 and AS-5-stored RBCs stored for 30, 42, 60, 80, 100 and 120 days. Though post-rejuvenation levels of ATP and 2,.3-DPG decreased with the storage duration of treated RBCs,

extremely long-stored RBCs were shown to be capable of a metabolism restart [95]. A study conducted by Koshkaryev *et al.* demonstrated the efficient effect of rejuvenation on reversal of stored RBCs adherence to endothelial cells, as well as lowered levels of membrane phosphatidyl serine (PS) exposure and intracellular Ca^{2+} and ROS [96]. Through the washing step, it is possible that fragilized older-RBCs suffer from hemolysis and can thus be selectively removed from the blood product (see below).

Pre-transfusion washing of long-stored RBCs

Recently, Cholette *et al.* conducted a prospective, randomized, clinical trial to investigate the washing of stored RBCs and platelets before transfusion to cardiopulmonary bypass surgery children patients [97]. It appeared obvious that such a pre-transfusion practice reduced inflammation markers. Though not significant, pre-transfusion washing tended to a reduced number of transfused blood units, and to a decreased mortality. In a more recent study, Bennett-Guerrero and coworkers compared different devices for washing of long-stored RBC units [98]. Over the removal of storage-related accumulating compounds (potassium, lactate), they found that the washing procedure can induce higher hemolysis and MV release. Free Hb and RBC-derived MVs are known to be scavengers of the vasodilator nitric oxide (NO), and are thus responsible for transfusion-induced impaired vascular function [99]. Recovered RBCs do not seem to be more sensitive to physical stress-induced hemolysis. On the contrary, filtration-related hemolysis appeared lowered on previously washed RBCs, presumably due to the fact that older erythrocytes already lysed during the washing process. Their results appeared to be washing device-dependent, of which the applied *g*-force seems to impact the quality of the final washed product [98]. A particular interest of RBCs washing is related to residual plasma in ECs. Depending on how the blood product is processed, from a few to 100 mL of anticoagulated plasma can be found in RBC units. In case of donor having anti-HLA Class II antibodies of high strength, recipient TRALI events can be attributed to the reactive residual plasma, as shown by Weber *et al.* [100]. Though time-consuming before transfusion, washing has the advantage of cleaning the blood products.

Oxygen-free RBCs storage

Oxidative lesions to proteins are linked with impaired cellular functions. As for the stored RBCs,

proteins involved in redox regulation, energy metabolism, and cytoskeleton organization are oxidatively challenged. To deal with the oxidative stress issue, research has been conducted towards an anaerobic storage of RBCs. In particular, Yoshida and coworkers brought promising results regarding the maintaining of 2,3-DPG and ATP levels, as well as in diminishing the number of MVs released by RBCs throughout the storage period. In a first study published in 2007, they applied 6 gas exchange cycles achieving a 50-fold reduction in free oxygen concentrations in RBC units [101]. Though impractical for routine EC storage, such setup showed interesting increased *in vitro* membrane stability below the conventional 6 weeks of storage in AS3 (reduced hemolysis and MV release rates). 2,3-DPG was completely depleted after 1 week of storage, whatever the oxygenation state. However, ATP preservation appeared better in anaerobic conditions, with initial level conserved up to 7 weeks, and an only 15 % decrease after 10 weeks (compared to the 64 % decrease in aerobic storage). The authors attributed this better ATP maintain to an increase of the glycolytic flux with a reduction of the pentose phosphate pathway, because of lower NADPH oxidation rate. In a second paper, Yoshida *et al.* investigated the pH of different AS, as well as a rejuvenation process, on anaerobically stored RBCs [102]. They showed that in anaerobic condition, the pH impacted ATP and 2,3-DPG preservation. Whatever the pH, both parameters appeared better maintained throughout the storage period in anaerobic conditions in comparison with aerobic storage. The pH seems particularly important for the preservation of 2,3-DPG in anaerobic environment since it was not impacted by oxygen depletion in RBCs stored in a more acidic pH AS (AS3, pH 5.8) [101]. The pH of AS appeared to have no effect on hemolysis, but a tendency to reduction of microvesiculation was observed in acidic anaerobic conditions [102]. Rejuvenation process after 7 weeks of acidic anaerobic storage allowed for a 24-hour recovery of 77.3 % of RBCs after 10 weeks of storage, with a low hemolysis (0.35 %). The 24-hour recovery was still acceptable for transfusion after 12 weeks of storage, providing that a second rejuvenation process was performed after 11 weeks. Similar improved parameters at current maximal storage duration and potential to prolong up to 9 weeks were shown on a novel AS, OFAS3 [103]. Additionally to these markers of RBCs storage and *in vivo* recovery data, D'Amici and colleagues investigated early storage-induced membrane proteins degradation and highlighted the role of oxygen

depletion in reducing such oxidative damages to proteins [104].

Hospital policies towards lowered number of blood transfusion

In general, blood transfusion services are facing a lowered demand from hospitals and physicians. The 2013 hemovigilance report from Swissmedic [31] highlighted a decrease of 6 % of delivered ECs for whole Switzerland. Between 2008 and 2013, the decrease rises up to 10.9 % (Figure 12). These data are in accordance with global new hospital policies regarding the transfusion of blood. For instance, this year, several papers published in Transfusion, the journal of the American Association of Blood Banks (AABB), dealt with hospital restrictive blood transfusion practices implemented in the USA. A retrospective study from the Roger Williams Hospital of Providence (Rhode Island) revealed significant lowered transfusion rates over a 9-year intervention period during which the ECs transfusion were cancelled or reduced based on patients Hb levels, in nonbleeding cases [105]. Interestingly, this decrease in RBC transfusion was closely correlated to a decrease of mortality ($r = 0.88$). Similarly, Yerrabothala and colleagues from the Dartmouth-Hitchcock Medical Center of Lebanon (New Hampshire) reported decreased number of transfused ECs thanks to local new policies, without change in length of stay nor in mortality rates [106]. Another example came from the Stanford Hospital & Clinics (California) where Goodnough and coworkers conducted a retrospective study of RBC transfusions and patient outcomes before and after the establishment of patient Hb level-based new transfusion policy [107]. Significantly decreased mortality, length of stay and 30-day readmission rates were determined thanks to the induced decline of transfusion rates.

In addition to lowered needs for RBCs blood products, works are being conducted to artificially produce red blood cells from culture of hematopoietic stem cells. The works of Luc Douay are very promising in this sense. Cultured RBCs were shown functional regarding deformability, enzyme content, Hb capacity to fix and release molecular oxygen and expression of blood group antigens [108]. In addition, cultured RBCs appeared to survive in vivo in human, presenting up to 63 % survival 26 days post-injection.

Even though these adaptations are reasonable, they will not cancel the need for blood donation. Artificial blood should not be soon commercially available, and natural blood will still

be transfused for many decades.

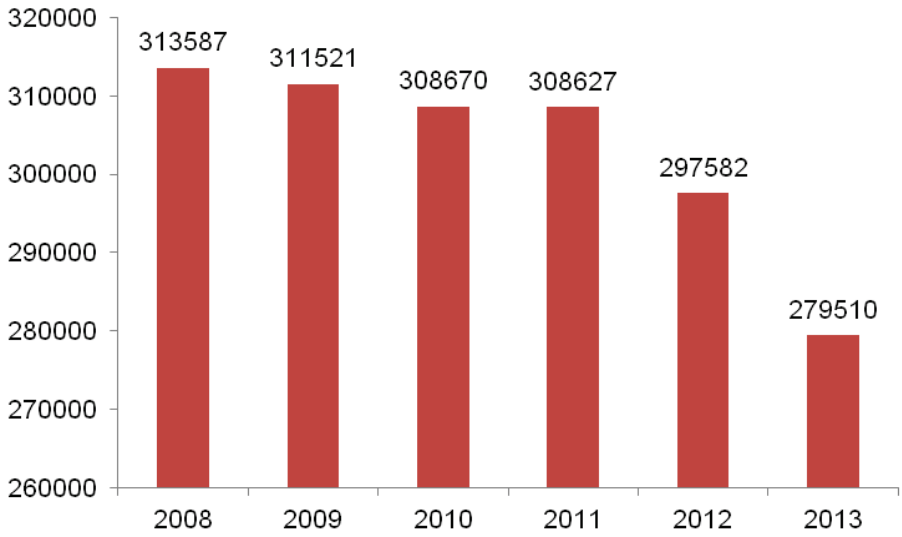


Figure 12: Number of ECs delivered in Switzerland from 2008 to 2013. Data published by Swissmedic in the 2013 hemovigilance annual report [31].

Objectives

Based on the present knowledge, a research project was designed at the Service Régional Vaudois de Transfusion Sanguine, aiming at tackling RBCs as they are stored for blood transfusion in Vaud, from a redox point of view. In a first step, the quantitation of storage-induced oxidative lesions to proteins will be presented. In particular, the oxidation of protein cysteine residues is investigated in cytoplasmic extracts of stored RBCs through an original 2D-DIGE-like approach, combining infrared labeling of reversibly oxidized proteins, 2D-electrophoresis separation, and densitometry quantitation. In addition, the carbonylation of proteins is addressed in 4 subcellular compartments: soluble Hb fraction, Hb-depleted soluble fraction, integral membrane protein fraction and cytoskeleton protein fraction. Carbonylated proteins from these different populations were quantified through chemical derivatization, 1D-electrophoresis separation, immunological detection and densitometry analysis. The goal of this part is to give more weight to the knowledge on occurrence of oxidative injuries to proteins in stored RBCs, by (1) investigating for the first time the occurrence of cysteine oxidation and trying to highlight different groups of proteins that would be differentially affected by this specific oxidative modification, and (2) specifically assessing subcellular protein populations to update and extend existing data on protein carbonylation.

In a second step, the present thesis will focus on the characterization of proteins that are affected by both oxidative modifications. Mass-spectrometry-driven identification of proteins of interest will give clues on how RBCs are affected by these oxidations. In particular, by investigating the function families of altered proteins, it should be possible to address whether such modifications occur in a targeted way or not. To this purpose, proteins presenting cysteine oxidation will be excised, digested and identified by LC-MS/MS and carbonylated proteins will be investigated by an affinity-capture strategy coupled to 1D-electrophoresis and LC-MS/MS identification. Gene ontology analyses will allow the study of protein functions engaged by oxidative lesions throughout the storage.

After the characterization of increasing oxidative injuries to proteins, the third step will regard

defense mechanisms that RBCs can implement to deal with oxidatively denatured proteins. This concerns (1) their proteolytic degradation by the 20S proteasome complexes, implying enzymatic assays for degradation of a specific fluorogenic substrate with samples of different storage durations, and (2) their elimination out of RBCs *via* the microvesiculation process, for which the quantitation of carbonylated protein content will be assessed, and its impact on cellular storage-related carbonylation evolution discussed.

Finally, the fourth part will conclude this PhD thesis, putting together each investigated points and picturing out an oxidative pathway model of the red blood cells storage. Efforts will be put on highlighting age-related storage damages, and an age limit for RBCs banking based on my investigations will be discussed.

References

1. Hughes-Jones, N.C. and B. Gardner, *Red cell agglutination: the first description by Creite (1869) and further observations made by Landois (1875) and Landsteiner (1901).* Br J Haematol, 2002. **119**(4): p. 889-93.
2. Ottenberg, R., *Studies in Isoagglutination : I. Transfusion and the Question of Intravascular Agglutination.* J Exp Med, 1911. **13**(4): p. 425-38.
3. Griesbach, H., *Beiträge zur Kenntniss des Blutes.* Archiv für die gesamte Physiologie des Menschen und der Tiere. **50**(9-10): p. 473-550.
4. Pekelharing, C.A., *Untersuchungen Über Das Fibrinferment*. 1892.
5. Wright, A.E., *On a Method of Determining the Condition of Blood Coagulability for Clinical and Experimental Purposes, and on the Effect of the Administration of Calcium Salts in Haemophilia and Actual or Threatened Haemorrhage: [Preliminary Communication]*. Br Med J, 1893. **2**(1700): p. 223-5.
6. Crile, G.W., *Hemorrhage and transfusion: an experimental and clinical research*. 1909: Appleton and Co.
7. Rous, P. and J.R. Turner, *The Preservation of Living Red Blood Cells in Vitro : I. Methods of Preservation.* J Exp Med, 1916. **23**(2): p. 219-37.
8. Rous, P. and J.R. Turner, *The Preservation of Living Red Blood Cells in Vitro : Ii. The Transfusion of Kept Cells.* J Exp Med, 1916. **23**(2): p. 239-48.
9. Robertson, O.H., *Transfusion with Preserved Red Blood Cells.* Br Med J, 1918. **1**(2999): p. 691-5.
10. Robertson, O.H., *A Method of Citrated Blood Transfusion.* Br Med J, 1918. **1**(2991): p. 477-9.
11. Misje, A.H., et al., *Motivation, recruitment and retention of voluntary non-remunerated blood donors: a survey-based questionnaire study.* Vox Sang, 2005. **89**(4): p. 236-44.
12. Levine, P. and R.E. Stetson, *Landmark article July 8, 1939. An unusual case of intra-group agglutination. By Philip Levine and Rufus E Stetson.* JAMA, 1984. **251**(10): p. 1316-7.
13. Landsteiner, K.W., A.S., *An Agglutinable Factor in Human Blood Recognized by Immune Sera for Rhesus Blood.* Society for Experimental Biology and Medicine, 1940. **43**(1): p. 223.
14. Landsteiner, K. and A.S. Wiener, *Studies on an agglutinogen (Rh) in human blood reacting with anti-Rhesus sera and with human isoantibodies.* J Exp Med, 1941. **74**(4): p. 309-20.
15. Cohn, E.J., et al., *Preparation and properties of serum and plasma proteins. I. Size and charge of proteins separating upon equilibration across membranes with ammonium sulfate solutions of controlled pH, ionic strength and temperature.* Journal of the American Chemical Society, 1940. **62**: p. 3386-3393.
16. Cohn, E.J., et al., *Preparation and properties of serum and plasma proteins. III. Size and charge of proteins separating upon equilibration across membranes with ethanol-water mixtures of controlled pH, ionic strength and temperature.* Journal of the American Chemical Society, 1940. **62**: p. 3396-3400.

17. Loutit, J.F., et al., *Citric acid-sodium citrate-glucose mixtures for blood storage.* Experimental Physiology, 1943. **32**(3): p. 183-202.
18. Gibson, J.G., 2nd, et al., *A cltrate-phosphatedextrose solution for the preservation of human blood.* Am J Clin Pathol, 1957. **28**(6): p. 569-78.
19. Gibson, J.G., 2nd, C.B. Gregory, and L.N. Button, *Citrate-phosphate-dextrose solution for preservation of human blood: a further report.* Transfusion, 1961. **1**: p. 280-7.
20. Walter, C.W. and W.P. Murphy, Jr., *A closed gravity technique for the preservation of whole blood in ACD solution utilizing plastic equipment.* Surg Gynecol Obstet, 1952. **94**(6): p. 687-92.
21. Artz, C.P., et al., *Plastic bags for intravenous infusions: observations in Korea with saline, dextran and blood*, in *Battle Casualties in Korea: Studies of the Surgical Research Team*. 1954, Army Medical Service Graduate School: Washington, DC. p. 219-224.
22. Hill, H.R., et al., *The effects of polyvinyl chloride and polyolefin blood bags on red blood cells stored in a new additive solution.* Vox Sang, 2001. **81**(3): p. 161-6.
23. Nakao, M., et al., *A new preservative medium containing adenine and inosine.* Bibl Haematol, 1962. **13**: p. 455-61.
24. Nakao, M., et al., *Effect of inosine and adenine on adenosine triphosphate regeneration and shape transformation in long-stored erythrocyts.* Biochim Biophys Acta, 1959. **32**: p. 564-5.
25. Nakao, M., T. Nakao, and S. Yamazoe, *Adenosine triphosphate and maintenance of shape of the human red cells.* Nature, 1960. **187**: p. 945-6.
26. Nakao, M., et al., *Adenosine triphosphate and shape of erythrocytes.* J Biochem, 1961. **49**: p. 487-92.
27. Nakao, K., et al., *A direct relationship between adenosine triphosphate-level and in vivo viability of erythrocytes.* Nature, 1962. **194**: p. 877-8.
28. Hogman, C.F., K. Hedlund, and H. Zetterstrom, *Clinical usefulness of red cells preserved in protein-poor mediums.* N Engl J Med, 1978. **299**(25): p. 1377-82.
29. Hogman, C.F., K. Hedlund, and Y. Sahlestrom, *Red cell preservation in protein-poor media. III. Protection against in vitro hemolysis.* Vox Sang, 1981. **41**(5-6): p. 274-81.
30. Yazer, M.H., et al., *The effect of prestorage WBC reduction on the rates of febrile nonhemolytic transfusion reactions to platelet concentrates and RBC.* Transfusion, 2004. **44**(1): p. 10-5.
31. Swissmedic, *Hémovigilance Rapport annuel 2013*. 2014.
32. Gossin, M., *Evaluation de la durée de stockage des concentrés érythrocytaires transfusés au CHUV*. 2014 (in preparation), UNIL: Lausanne.
33. Chiu, P., P.D. Roy, and J.C. Marshall, *Blood transfusion is a risk factor for ICU-acquired infection and the multiple organ dysfunction syndrome (MODS).* Critical Care Medicine, 1993. **21**(4): p. S226.
34. Marik, P.E. and H.L. Corwin, *Efficacy of red blood cell transfusion in the critically ill: a systematic review of the literature.* Crit Care Med, 2008. **36**(9): p. 2667-74.
35. Marik, P.E. and W.J. Sibbald, *Effect of stored-blood transfusion on oxygen delivery in patients with sepsis.* JAMA, 1993. **269**(23): p. 3024-9.
36. Purdy, F.R., M.G. Tweeddale, and P.M. Merrick, *Association of mortality with age of blood transfused in septic ICU patients.* Can J Anaesth, 1997. **44**(12): p. 1256-61.

37. Vamvakas, E.C. and J.H. Carven, *Transfusion and postoperative pneumonia in coronary artery bypass graft surgery: effect of the length of storage of transfused red cells.* Transfusion, 1999. **39**(7): p. 701-10.
38. Jensen, L.S., et al., *Randomised comparison of leucocyte-depleted versus buffy-coat-poor blood transfusion and complications after colorectal surgery.* The Lancet, 1996. **348**(9031): p. 841-845.
39. van de Watering, L.M., et al., *Beneficial effects of leukocyte depletion of transfused blood on postoperative complications in patients undergoing cardiac surgery: a randomized clinical trial.* Circulation, 1998. **97**(6): p. 562-8.
40. Bordin, J.O., N.M. Heddle, and M.A. Blajchman, *Biologic effects of leukocytes present in transfused cellular blood products.* Blood, 1994. **84**(6): p. 1703-21.
41. Phelan, H.A., et al., *Prestorage leukoreduction abrogates the detrimental effect of aging on packed red cells transfused after trauma: a prospective cohort study.* Am J Surg, 2012. **203**(2): p. 198-204.
42. Antonelou, M.H., et al., *Effects of pre-storage leukoreduction on stored red blood cells signaling: a time-course evaluation from shape to proteome.* J Proteomics, 2012. **76 Spec No.**: p. 220-38.
43. Sanders, J., et al., *Red blood cell storage is associated with length of stay and renal complications after cardiac surgery.* Transfusion, 2011. **51**(11): p. 2286-94.
44. Zallen, G., et al., *Age of transfused blood is an independent risk factor for postinjury multiple organ failure.* Am J Surg, 1999. **178**(6): p. 570-2.
45. Vincent, J.L., et al., *Anemia and blood transfusion in critically ill patients.* JAMA, 2002. **288**(12): p. 1499-507.
46. Leal-Noval, S.R., et al., *Influence of erythrocyte concentrate storage time on postsurgical morbidity in cardiac surgery patients.* Anesthesiology, 2003. **98**(4): p. 815-22.
47. Rao, S.V., et al., *Relationship of blood transfusion and clinical outcomes in patients with acute coronary syndromes.* JAMA, 2004. **292**(13): p. 1555-62.
48. Kuduvalli, M., et al., *Effect of peri-operative red blood cell transfusion on 30-day and 1-year mortality following coronary artery bypass surgery.* Eur J Cardiothorac Surg, 2005. **27**(4): p. 592-8.
49. Koch, C.G., et al., *Duration of red-cell storage and complications after cardiac surgery.* N Engl J Med, 2008. **358**(12): p. 1229-39.
50. Katsios, C., et al., *Red blood cell transfusion and increased length of storage are not associated with deep vein thrombosis in medical and surgical critically ill patients: a prospective observational cohort study.* Crit Care, 2011. **15**(6): p. R263.
51. McKenny, M., et al., *Age of transfused blood is not associated with increased postoperative adverse outcome after cardiac surgery.* Br J Anaesth, 2011. **106**(5): p. 643-9.
52. Kekre, N., et al., *Storage time of transfused red blood cells and impact on clinical outcomes in hematopoietic stem cell transplantation.* Transfusion, 2011. **51**(11): p. 2488-94.
53. Dunn, L.K., et al., *Duration of red blood cell storage and outcomes following orthotopic liver transplantation.* Liver Transpl, 2012. **18**(4): p. 475-81.
54. Saager, L., et al., *Erythrocyte storage duration is not associated with increased mortality in noncardiac surgical patients: a retrospective analysis of 6,994 patients.* Anesthesiology, 2013. **118**(1): p. 51-8.

55. Manlhiot, C., et al., *Longer blood storage is associated with suboptimal outcomes in high-risk pediatric cardiac surgery.* Ann Thorac Surg, 2012. **93**(5): p. 1563-9.
56. van de Watering, L.M., *Age of blood: does older blood yield poorer outcomes?* Curr Opin Hematol, 2013. **20**(6): p. 526-32.
57. D'Alessandro, A., et al., *Time-course investigation of SAGM-stored leukocyte-filtered red bood cell concentrates: from metabolism to proteomics.* Haematologica, 2012. **97**(1): p. 107-15.
58. Hess, J.R., *Red cell changes during storage.* Transfus Apher Sci, 2010. **43**(1): p. 51-9.
59. Bennett-Guerrero, E., et al., *Evolution of adverse changes in stored RBCs.* Proc Natl Acad Sci U S A, 2007. **104**(43): p. 17063-8.
60. Roback, J.D., et al., *Metabolomics of ADSOL (AS-1) red blood cell storage.* Transfus Med Rev, 2014. **28**(2): p. 41-55.
61. Mendiratta, S., Z.C. Qu, and J.M. May, *Erythrocyte ascorbate recycling: antioxidant effects in blood.* Free Radic Biol Med, 1998. **24**(5): p. 789-97.
62. Giustarini, D., et al., *Red blood cells as a physiological source of glutathione for extracellular fluids.* Blood Cells Mol Dis, 2008. **40**(2): p. 174-9.
63. Misra, H.P. and I. Fridovich, *The generation of superoxide radical during the autoxidation of hemoglobin.* J Biol Chem, 1972. **247**(21): p. 6960-2.
64. Cimen, M.Y., *Free radical metabolism in human erythrocytes.* Clin Chim Acta, 2008. **390**(1-2): p. 1-11.
65. Sies, H., *Strategies of antioxidant defense.* Eur J Biochem, 1993. **215**(2): p. 213-9.
66. Dumaswala, U.J., et al., *Protein and lipid oxidation of banked human erythrocytes: role of glutathione.* Free Radic Biol Med, 1999. **27**(9-10): p. 1041-9.
67. D'Alessandro, A., et al., *An update on red blood cell storage lesions, as gleaned through biochemistry and omics technologies.* Transfusion, 2014.
68. Delobel, J., et al., *Subcellular fractionation of stored red blood cells reveals a compartment-based protein carbonylation evolution.* J Proteomics, 2012. **76 Spec No.**: p. 181-93.
69. Kriebardis, A.G., et al., *Membrane protein carbonylation in non-leukodepleted CPDA-preserved red blood cells.* Blood Cells Mol Dis, 2006. **36**(2): p. 279-82.
70. Kriebardis, A.G., et al., *Progressive oxidation of cytoskeletal proteins and accumulation of denatured hemoglobin in stored red cells.* J Cell Mol Med, 2007. **11**(1): p. 148-55.
71. Low, P.S., et al., *Characterization of the reversible conformational equilibrium of the cytoplasmic domain of erythrocyte membrane band 3.* J Biol Chem, 1984. **259**(21): p. 13070-6.
72. Waugh, S.M. and P.S. Low, *Hemichrome binding to band 3: nucleation of Heinz bodies on the erythrocyte membrane.* Biochemistry, 1985. **24**(1): p. 34-9.
73. Waugh, S.M., J.A. Walder, and P.S. Low, *Partial characterization of the copolymerization reaction of erythrocyte membrane band 3 with hemichromes.* Biochemistry, 1987. **26**(6): p. 1777-83.
74. Hornig, R. and H.U. Lutz, *Band 3 protein clustering on human erythrocytes promotes binding of naturally occurring anti-band 3 and anti-spectrin antibodies.* Exp Gerontol, 2000. **35**(8): p. 1025-44.
75. Kay, M.M., et al., *Alteration in membrane protein band 3 associated with accelerated erythrocyte aging.* Proc Natl Acad Sci U S A, 1989. **86**(15): p. 5834-8.

76. Lutz, H.U., R. Flepp, and G. Stringaro-Wipf, *Naturally occurring autoantibodies to exoplasmic and cryptic regions of band 3 protein, the major integral membrane protein of human red blood cells.* J Immunol, 1984. **133**(5): p. 2610-8.
77. Lutz, H.U. and G. Stringaro-Wipf, *Senescent red cell-bound IgG is attached to band 3 protein.* Biomed Biochim Acta, 1983. **42**(11-12): p. S117-21.
78. Kay, M.M., *Localization of senescent cell antigen on band 3.* Proc Natl Acad Sci U S A, 1984. **81**(18): p. 5753-7.
79. Kay, M.M., et al., *Senescent cell antigen is immunologically related to band 3.* Proc Natl Acad Sci U S A, 1983. **80**(6): p. 1631-5.
80. Kay, M.M., T. Wyant, and J. Goodman, *Autoantibodies to band 3 during aging and disease and aging interventions.* Ann N Y Acad Sci, 1994. **719**: p. 419-47.
81. Lutz, H.U., P. Stammler, and S. Fasler, *How naturally occurring anti-band 3 antibodies stimulate C3b deposition to senescent and oxidatively stressed red blood cells.* Biomed Biochim Acta, 1990. **49**(2-3): p. S224-9.
82. Terpstra, V. and T.J. van Berkel, *Scavenger receptors on liver Kupffer cells mediate the in vivo uptake of oxidatively damaged red blood cells in mice.* Blood, 2000. **95**(6): p. 2157-63.
83. Schroit, A.J., J.W. Madsen, and Y. Tanaka, *In vivo recognition and clearance of red blood cells containing phosphatidylserine in their plasma membranes.* J Biol Chem, 1985. **260**(8): p. 5131-8.
84. Hovav, T., et al., *Alteration of red cell aggregability and shape during blood storage.* Transfusion, 1999. **39**(3): p. 277-81.
85. Rubin, O., et al., *Microparticles in stored red blood cells: an approach using flow cytometry and proteomic tools.* Vox Sang, 2008. **95**(4): p. 288-97.
86. Frank, S.M., et al., *Decreased erythrocyte deformability after transfusion and the effects of erythrocyte storage duration.* Anesth Analg, 2013. **116**(5): p. 975-81.
87. Relevy, H., et al., *Blood banking-induced alteration of red blood cell flow properties.* Transfusion, 2008. **48**(1): p. 136-46.
88. Berezina, T.L., et al., *Influence of storage on red blood cell rheological properties.* J Surg Res, 2002. **102**(1): p. 6-12.
89. Willekens, F.L., et al., *Erythrocyte vesiculation: a self-protective mechanism?* Br J Haematol, 2008. **141**(4): p. 549-56.
90. Bosman, G.J., et al., *The proteome of red cell membranes and vesicles during storage in blood bank conditions.* Transfusion, 2008. **48**(5): p. 827-35.
91. Hess, J.R., et al., *Successful storage of RBCs for 9 weeks in a new additive solution.* Transfusion, 2000. **40**(8): p. 1007-11.
92. Hess, J.R., et al., *Successful storage of RBCs for 10 weeks in a new additive solution.* Transfusion, 2000. **40**(8): p. 1012-6.
93. Hess, J.R., et al., *RBC storage for 11 weeks.* Transfusion, 2001. **41**(12): p. 1586-90.
94. Hess, J.R., et al., *Twelve-week RBC storage.* Transfusion, 2003. **43**(7): p. 867-72.
95. Meyer, E.K., et al., *Rejuvenation capacity of red blood cells in additive solutions over long-term storage.* Transfusion, 2011. **51**(7): p. 1574-9.
96. Koshkaryev, A., et al., *Rejuvenation treatment of stored red blood cells reverses storage-induced adhesion to vascular endothelial cells.* Transfusion, 2009. **49**(10): p. 2136-43.

97. Cholette, J.M., et al., *Washing red blood cells and platelets transfused in cardiac surgery reduces postoperative inflammation and number of transfusions: results of a prospective, randomized, controlled clinical trial.* Pediatr Crit Care Med, 2012. **13**(3): p. 290-9.
98. Bennett-Guerrero, E., et al., *Randomized study of washing 40- to 42-day-stored red blood cells.* Transfusion, 2014.
99. Donadee, C., et al., *Nitric oxide scavenging by red blood cell microparticles and cell-free hemoglobin as a mechanism for the red cell storage lesion.* Circulation, 2011. **124**(4): p. 465-76.
100. Weber, L.L., L.D. Roberts, and J.D. Sweeney, *Residual plasma in red blood cells and transfusion-related acute lung injury.* Transfusion, 2014.
101. Yoshida, T., et al., *Extended storage of red blood cells under anaerobic conditions.* Vox Sang, 2007. **92**(1): p. 22-31.
102. Yoshida, T., et al., *The effects of additive solution pH and metabolic rejuvenation on anaerobic storage of red cells.* Transfusion, 2008. **48**(10): p. 2096-105.
103. Dumont, L.J., T. Yoshida, and J.P. AuBuchon, *Anaerobic storage of red blood cells in a novel additive solution improves in vivo recovery.* Transfusion, 2009. **49**(3): p. 458-64.
104. D'Amici, G.M., S. Rinalducci, and L. Zolla, *Proteomic analysis of RBC membrane protein degradation during blood storage.* J Proteome Res, 2007. **6**(8): p. 3242-55.
105. Tavares, M.M., P.J. Diquattro, and J.D. Sweeney, *Reduction in red blood cell transfusion associated with engagement of the ordering physician.* Transfusion, 2014.
106. Yerrabothala, S., et al., *Significant reduction in red blood cell transfusions in a general hospital after successful implementation of a restrictive transfusion policy supported by prospective computerized order auditing.* Transfusion, 2014.
107. Goodnough, L.T., et al., *Restrictive blood transfusion practices are associated with improved patient outcomes.* Transfusion, 2014.
108. Giarratana, M.C., et al., *Proof of principle for transfusion of in vitro-generated red blood cells.* Blood, 2011. **118**(19): p. 5071-9.

PART 2.

QUANTITATION OF HALLMARKS OF PROTEIN OXIDATIVE DAMAGES DURING THE STORAGE OF ERYTHROCYTE CONCENTRATES

Table of contents

List of illustrations

List of equations

Context

Among all the storage lesions described so far (see Part 1), a particular interest is put on the oxidative-derived ones, since such damages cannot be reversed through transfusion processes, contrarily to some morphological or metabolism modifications, such as ATP level that is recovered after a few hours post-transfusion [1, 2]. Since erythrocytes are unable to perform protein synthesis, no protein turnover is possible. Damaged proteins should accumulate until being degraded or eliminated out of the cell. Redox proteomic studies performed on stored RBCs have shown increasing hallmark of oxidative stress throughout the storage period. Two main oxidative protein modifications, the oxidation of cysteine residues and the protein carbonylation, will be investigated here. Additionally to collecting data relative to protein oxidation in stored erythrocytes, this part of my research intends to bring new comprehensive information about RBCs aging in blood banking conditions, as well as high importance clues potentially regarding causes of transfusion-related adverse outcomes, to the blood transfusion community.

Chapter one

Evolution of protein cysteine oxidation

This chapter refers to the article

"Cysteine redox proteomics of the hemoglobin-depleted cytosolic fraction of stored red blood cells"

In preparation

Introduction

Protein oxidation is widely investigated through the cysteine redox status. Disulfide bridge formation (inter- or intra-molecular), mixed disulfides involving cysteines and low-molecular weight thiols (*e.g.* S-glutathionylation or S-cysteinylation), and sulfenic acid, can be transient oxidation states involved in processes regulating protein functions. It is particularly the case of several enzymes (*e.g.* peroxiredoxin, thioredoxin, glutaredoxin, glutathione peroxidase, catalase or superoxide dismutase), as well as small molecules (*e.g.* glutathione, cysteine, vitamin C or vitamin E) involved in anti-oxidant defenses of the RBC. Quite recently, it has also been demonstrated that the sulfinic acid (double oxidation of cysteine) can be reduced by sulfiredoxin, enabling the re-activation of peroxiredoxins [3, 4]. Alterations of such proteins and molecules at thiol moieties, known to be highly prone to oxidation, can lead to the RBCs incapacity to protect themselves against reactive oxygen species (ROS), amplifying their deleterious effects.

Few methods are available to study thiol oxidative states, most of them being gel-based approaches coupled to mass spectrometry identification. Disulfides have been detected by diagonal electrophoresis (consisting in a first non-reducing SDS-PAGE followed by a second reducing one, most protein spots being on a diagonal, and proteins detected upper or lower the diagonal present intra- or inter-molecular disulfide bridges, respectively) [5-7]. Disulfide proteins have also been analyzed by 2DE using labeling with fluorescent or radioactive derivatives of alkylating reagent iodoacetamide [8, 9]. Proteins susceptible to glutathionylation have been revealed or purified after labeling with biotin-coupled oxidized glutathione (R-S-SG-Biot), either by western blot using streptavidin-HRP or by affinity chromatography using streptavidin-coupled sepharose beads [10]. Nitrosylated-cysteines and sulfenic acids can be reduced specifically by ascorbic acid [11] and arsenite [12], respectively, and then labeled by biotin-derivatives for detection [11-13]. These approaches are specific to some oxidative-related modifications, and do not assess all thiol-related oxidations. Such approach has been made feasible since the development of 2D-DIGE and the use of amine reactive dyes [14]. Applied to cysteines, the redox-DIGE uses fluorescent labeling of cysteines based on thiol reactivity. Coupled to MS identification, redox-DIGE appears to be a technique of choice for identification

and quantitation of redox proteomes [15, 16]. This involves sequential alkylation, reduction and labeling of thiol groups. Depending on the order at which these reactions are performed, one can label and visualize either free cysteines or oxidized ones (for a review, see [17]). Moreover, the availability of different probes allows mixing differently-labeled samples and visualizing proteins on only one 2D gel, getting rid of the low reproducibility of this separation technique. It enables the direct comparison of either reduced *versus* oxidized cysteines or oxidized cysteines in different samples or different conditions.

To the best of our knowledge, the evolution of cysteine oxidation in RBCs throughout their banking as erythrocyte concentrates (ECs) has never been studied so far. A particular interest has risen on protein glutathionylation since the early 2000's, which is now known to transiently modulate protein functions [18, 19]. As for RBCs [20], this modification has been shown to be involved in some pathophysiological processes [21-23]. Also, oxidative stress-induced membrane and cytoskeletal protein glutathionylation in erythrocytes was correlated with decrease in membrane deformability and stability [24-27].

Study design

Five independent erythrocyte concentrates (namely EC1, EC2, EC3, EC4 and EC5) were followed during the 42-days storage period. Some details about the donors can be found in annex A. Samples were collected at three time-points during the storage period, giving access to the cysteine oxidation status at an early storage duration (sample "day 6"), at the middle (sample "day 27") and at the end (sample "day 41") of the storage period. This study focused on the erythrocyte soluble fraction, since many redox-related proteins, whose mechanisms of action rely on the oxidation status of a cysteine, are found in the cytoplasm of RBCs. A 2D-DIGE-like approach based on labeling of oxidized proteins with infrared dyes, developed by Riederer's group [28], was applied here in order to make direct two-by-two comparisons of samples from each EC. The effect of the storage period on the oxidation of cysteines was evaluated by comparing the infrared (IR) images of labeled proteins thanks to dedicated software. The study design is graphically described in Figure 13.

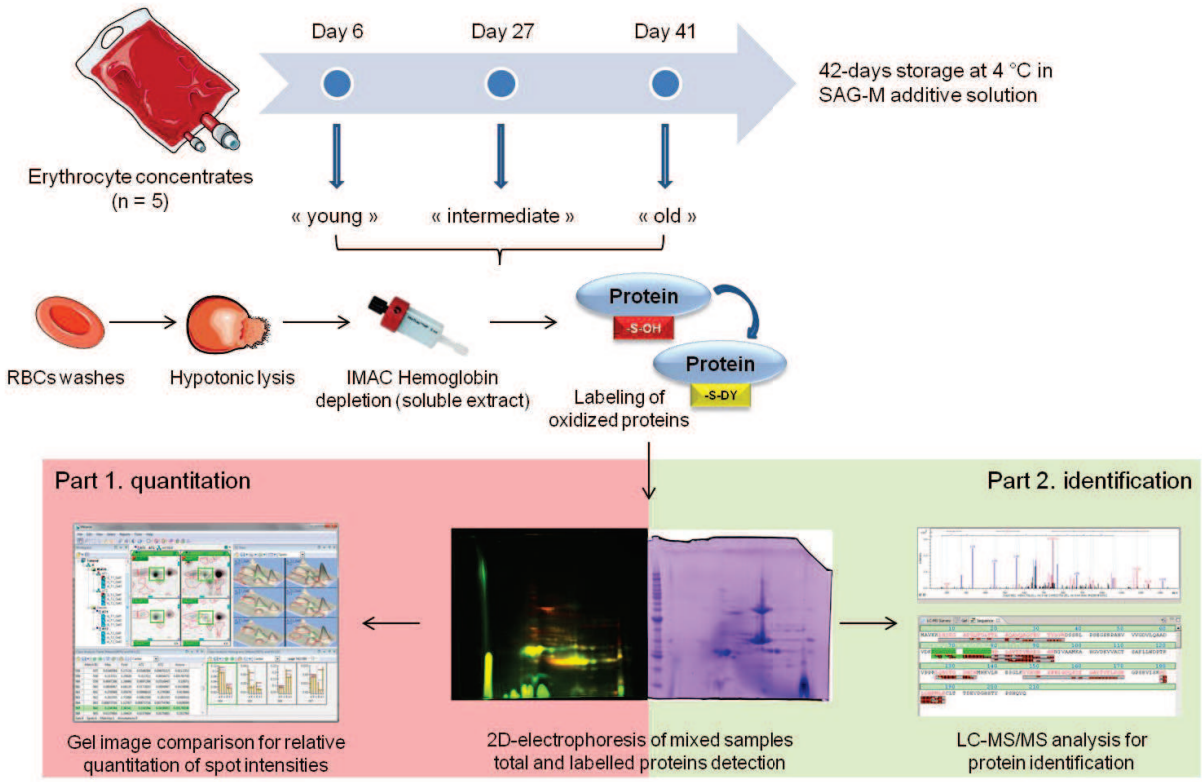


Figure 13: Study design for the characterization of cysteine oxidation in soluble extracts of stored RBCs.

Material and methods

Sample collection and preparation

Each day of sample collection, around 15 mL of each EC were centrifuged at 2000 *g* during 10 minutes and at 4 °C to remove additive solution and hemolysis-derived components. Then, RBCs were washed three times by resuspending the cellular pellets in physiological 0.9 % NaCl solution, and centrifugation in the same conditions as before. Depending on the hemolysis rate, that is higher for end-storage ECs, additional washing steps were performed until obtaining a clear washing supernatant, free of residual Hb. Washed RBCs were lyzed by a one hour incubation with two pellet volumes of a 0.1x PBS solution supplemented with a mixture of anti-proteases (1 Complete protease inhibitor cocktail tablet per 50 mL of lysis buffer), under agitation at 4 °C. RBC soluble cytoplasmic extracts were finally separated from membranes by ultracentrifugation at 100'000 *g* for 30 minutes at 4 °C.

RBC soluble extracts were depleted of Hb through nickel-based metal ion affinity chromatography using a 5 mL HisTrap column, on a low pressure chromatography system (Biologic Workstation, BIO-RAD, Hercules, CA, USA), based on reference [29]. Each flow-through, corresponding to the RBC Hb-depleted soluble extracts, was collected as a single fraction upon detection of the protein 280 nm UV signal. This depletion step, even if not technically required, was implemented due to the important dynamic range between Hb and other proteins.

Protein concentrations were determined using the Bradford Protein Assay (BIO-RAD, Hercules, CA, USA) based on reference [30].

Oxidized cysteines labeling

The labeling of oxidized-cysteines, schematized in Figure 14, was designed as follow: first, free cysteines from 500 µg of proteins were blocked by alkylation with 8 mM N-ethylmaleimide (NEM) in 1/1000 Tween 20-containing PBS solution, overnight at 4 °C under gentle shaking. Excess of NEM was then removed by filtration over a D-salt dextrane desalting column (Thermo, Rockford, IL, USA), and proteins were recovered in several fractions. Afterwards, high protein content-fractions were detected by dot assays, pooled, and concentrated using Vivaspin ultrafiltration spin columns (Sartorius AG, Goettingen, Germany) with a MW cut off of 5000 Da. Secondly, cysteines engaged in disulfide bridges (R-S-S-R) as well as reversibly oxidized cysteines (R-SOH sufenic acid and R-SO_2H sulfinic acid states) were reduced by incubation with 21 mM 2-mercaptoethylamine (2-MEA) in 0.1 M sodium phosphate dibasic, 2.5 mM EDTA reducing buffer, for 90 minutes at 37 °C under gentle shaking and protected from light. Then, samples were desalted and high protein content-fractions were pooled and concentrated as before, except that the desalting buffer contained 1 mM EDTA. Finally, newly free cysteines were labeled by incubation with 20 ng of thiol-reactive infrared DY-680 or DY-780 maleimides, revealed as red and green IR signals, respectively. For each EC followed, the day 6 samples were labeled with the DY-680 maleimide whereas the day 27 and day 41 samples were labeled with the DY-780 maleimide. Labeled samples were again desalted, and high protein content-fractions were pooled and concentrated, and protein concentrations were determined using the Bradford Protein Assay. Samples were stored at -20 °C, protected from light, until used.

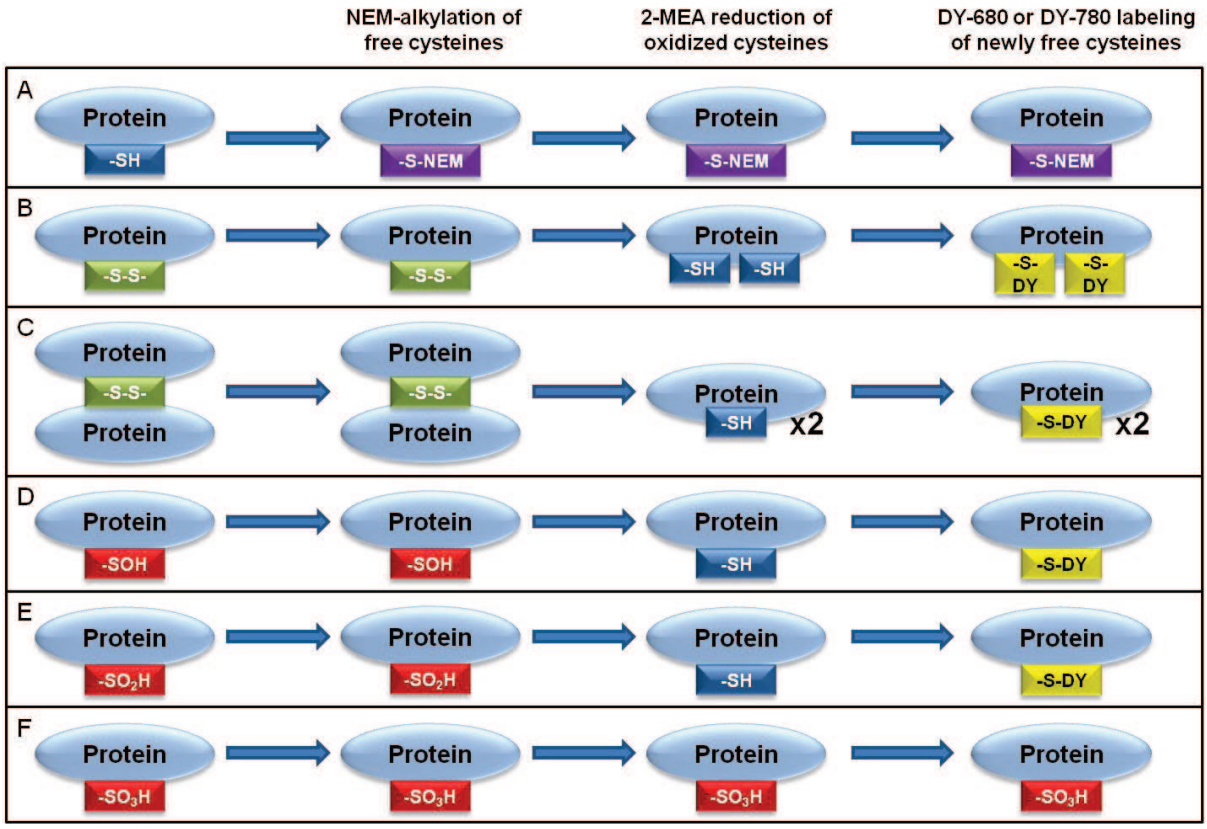


Figure 14: Labeling of different cysteine thiol states. The behavior to labeling process is depicted for free cysteines (A), intra-molecular disulfide bridge (B), inter-molecular disulfide bridge (C), sulfenic (D), sulfinic (E) and sulfonic (F) acid states.

2D gel electrophoresis

In order to compare the three storage durations, samples were mixed two by two at a total of 150 µg, for each EC. The day 6 sample was taken as the reference and was mixed either with the day 27 sample or with the day 41 one. Mixed samples were resuspended in 125 µL of rehydration buffer composed of 7 M urea, 2 M thiourea, 4 % CHAPS, 1 % DTE, 0.8 % carrier ampholytes, and traces of bromophenol blue. Proteins were separated on a first dimension according to their isoelectric point (*pI)* through isoelectric-focusing on 7 cm 4-10 Immobilized pH Gradient (IPG) strips (GE Healthcare, Fairfield, CT, USA). Once the IEF finished, the strips were first incubated during 15 minutes with gentle shaking in 6 M Urea, 8.75 mM Tris-HCl, 30 % (v/v) glycerol and 0.002% Bromophenol Blue equilibration buffer supplemented with 0.1 mM DTE, then during 15 minutes in equilibration buffer supplemented with 125 mM IAA. IPG strips were then loaded onto precast SDS-PAGE gels (Mini-PROTEAN TGX Gels 4-15 %, BIO-RAD,

Hercules, CA, USA) for 2DE, sealed with 0.5 % agarose, and proteins were separated on a second dimension according to their MW, through SDS-PAGE, at constant 35 mA per gel. 5 µL of BenchMark Protein Ladder (Life Technologies Europe B.V., Zug, Switzerland) were also loaded on each gel in a separate well. Proteins were finally revealed using the ODYSSEY® CLx infrared imaging system (LI-COR, Lincoln, NE, USA), allowing excitation at 685 nm and 785 nm through its "700" and "800" channel laser sources, respectively. The two images from the two 2D gels (two sample mixes) of each EC were acquired simultaneously for easy comparison of both the two "700" patterns and the two "800" patterns (same acquisition parameters). It is thus possible to visualize labeled proteins originating from only one sample of the mix, from the other sample, or from both samples. Basically, samples labeled with the DY-680 maleimide are visualized in red and samples labeled with the DY-780 maleimide are visualized in green. Once the IR images obtained, the 2D gels were stained for total protein labeling with Coomassie Blue, and images were acquired using a classical gel scanner.

Image analysis

The mixed images showing both DY-680- and DY-780-labeled proteins allow comparing the oxidative status of cysteine-containing proteins (as shown in Figure 14 and Figure 15) along the storage period of the five ECs followed. Spots appearing in yellow correspond to proteins presenting reversible cysteine oxidation in both conditions (two storage durations), whereas spots appearing mostly in red, or mostly in green correspond to proteins presenting more cysteine oxidation in the first (day 6) or the second (day 27 or day 41) condition, respectively. Spots of interest can thus be directly detected this way.

For each of the five ECs, the IR patterns of four 2D gel images were compared: a first day 6 pattern corresponding to the mix [day 6 + day 27], the second day 6 pattern corresponding to the mix [day 6 + day 41], the day 27 pattern and the day 41 pattern. Spots were semi-automatically detected and matched among the five sets of four images via the ImageMaster™ 2D Platinum 7 software (version 7.0.6, GE Healthcare, Fairfield, CT, USA). Spots successfully matched between at least four of the five sets were analyzed to compare their intensities, expressed as "volume %", that is to say the percentage that each spot volume represents over the total volume of all detected spots in the same gel. The "volume %" of spots of the day 6

sample, common to both sample mixes of each EC, allowed data normalization.

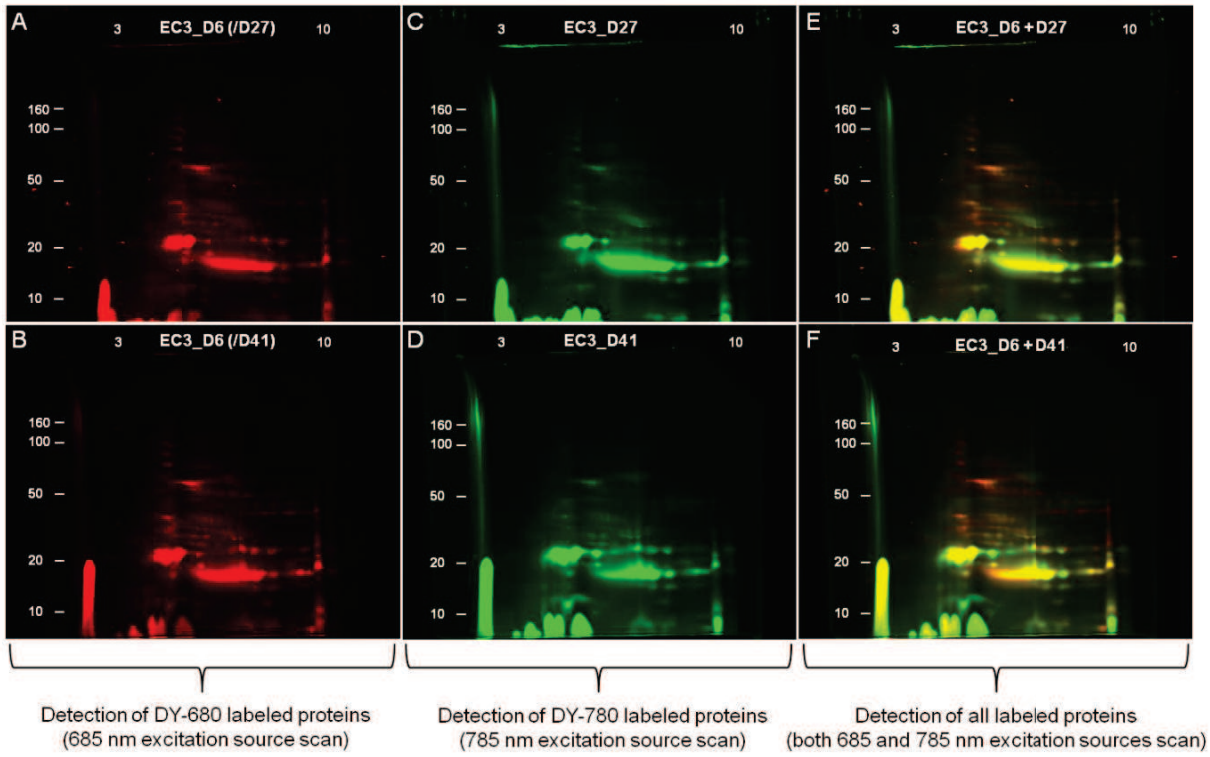


Figure 15: Representative 2D gel images after IR detection of labeled proteins. Six 2D gel images of infrared detection can be obtained per EC, corresponding to the two sample mixes [Day 6 + Day 27] (A, C and E) or [Day 6 + Day 41] (B, D and F): in red the patterns of labeled proteins in the Day 6 sample (A and B), in green the patterns of Day 27 (C) or Day 41 (D) samples, and in yellow the two corresponding mixed images (E and F).

Data processing and statistics

Spot intensities at days 27 and 41 were normalized relatively to the corresponding day 6 intensities for each of the five ECs. Then, the mean normalized intensities were used to classify spots in four categories depending on their evolution over the storage period, *i.e.* mean increasing tendency (referred to as “up” category), mean decreasing tendency (referred to as “down” category), up peak pattern at day 27 (referred to as “mid-up” category) and down peak pattern at day 27 (referred to as “mid-down” category). Spots at days 27 or 41 were considered of lower or higher intensity in comparison to the day 6 if mean fold changes were below 0.8 or above 1.2, respectively. In the same way, intensity ratio between days 27 and 41 was calculated

according to Equation 1.

Statistics were performed using the free open source RStudio IDE software (Version 0.97.551). The significance of spot segregation in four categories was checked by one-way Analysis of Variance (ANOVA) followed by a Tukey HSD test for pairwise multiple comparisons. The influence of storage duration on spot intensity was investigated the same way in each category.

$$R(days\ 41{,}27) = I'(day\ 41)/I'(day\ 27) = \frac{I(day\ 41)}{I(day\ 6{,}41)} \Big/ \frac{I(day\ 27)}{I(day\ 6{,}27)}$$

Equation 1. Determination of spot intensity ratio. $\mathbf{I'(day\ X)}$ is the normalized intensity of a spot at day X, $\mathbf{I(day\ X)}$ is the raw intensity of the same spot at day X, and $\mathbf{I(day\ 6, X)}$ is the intensity of the same spot from sample "day 6" corresponding to the mix [day 6 + day X].

Results and discussion

Infrared detection of 2D gels revealed lot of labeled proteins, even in the day 6 samples, as it can be seen in Figure 15A and B. This means that many proteins present a reversible oxidation state at their cysteine residue(s), early in the storage period. It is known that the oxidation state of cysteines can be determinant for protein structure and function (for instance, intra-molecular disulfide bridges are sometimes required for the proper folding of proteins, and misfolding can lead to loss of function) and that redox variation of cysteines is involved in several physiological processes, thus naturally occurring independently of any stress condition, as for instance the *ex vivo* storage and aging of RBCs. Differences of labeled proteome 2DE patterns were observed according to the storage duration (see Figure 15), implying an evolution of cysteine oxidation in a storage time-dependent manner, in addition to the basal physiological cysteine redox states. In particular, the densitometric analysis of the 20 2D gel images highlighted 32 spots that were successfully matched among the four images (2x Day6 + Day 27 + Day 41) of at least four out of the five ECs. An important inter-sample variability was observed, in term of intensity ratio, and even sometimes revealing different pattern evolution for a same spot. Thus, mean relative abundances were here associated with relatively high standard deviation. A mean coefficient of variation of 55 % was thus calculated over the 32 spots. This value was similar whatever the mean evolution tendency of the spots.

The 32 spots of interest were classified in four categories, depending on their intensity evolution throughout the storage period (see Figure 16 and Figure 17). This segregation based on mean standardized intensities was proven statistically significant by one-way ANOVA ($p < 5 \cdot 10^{-10}$ for the global data set, $p < 5 \cdot 10^{-6}$ at day 27 and $p < 1 \cdot 10^{-8}$ at day 41, see Figure 16 and Figure 17). Test for multiple comparisons further confirmed this, showing significant differences between categories “up” and “down” ($p < 5 \cdot 10^{-5}$), “mid-up” and “down” ($p < 0.001$), “mid-up” and “mid-down” ($p < 0.01$) and “up” and “mid-down” ($p < 5 \cdot 10^{-4}$) at day 27 (see Figure 16A), and between categories “up” and “down” ($p < 10^{-7}$), “up” and “mid-down” ($p < 0.005$), “up” and “mid-up” ($p < 10^{-5}$) and “mid-down” and “down” ($p < 0.05$) at day 41 (see Figure 16B).

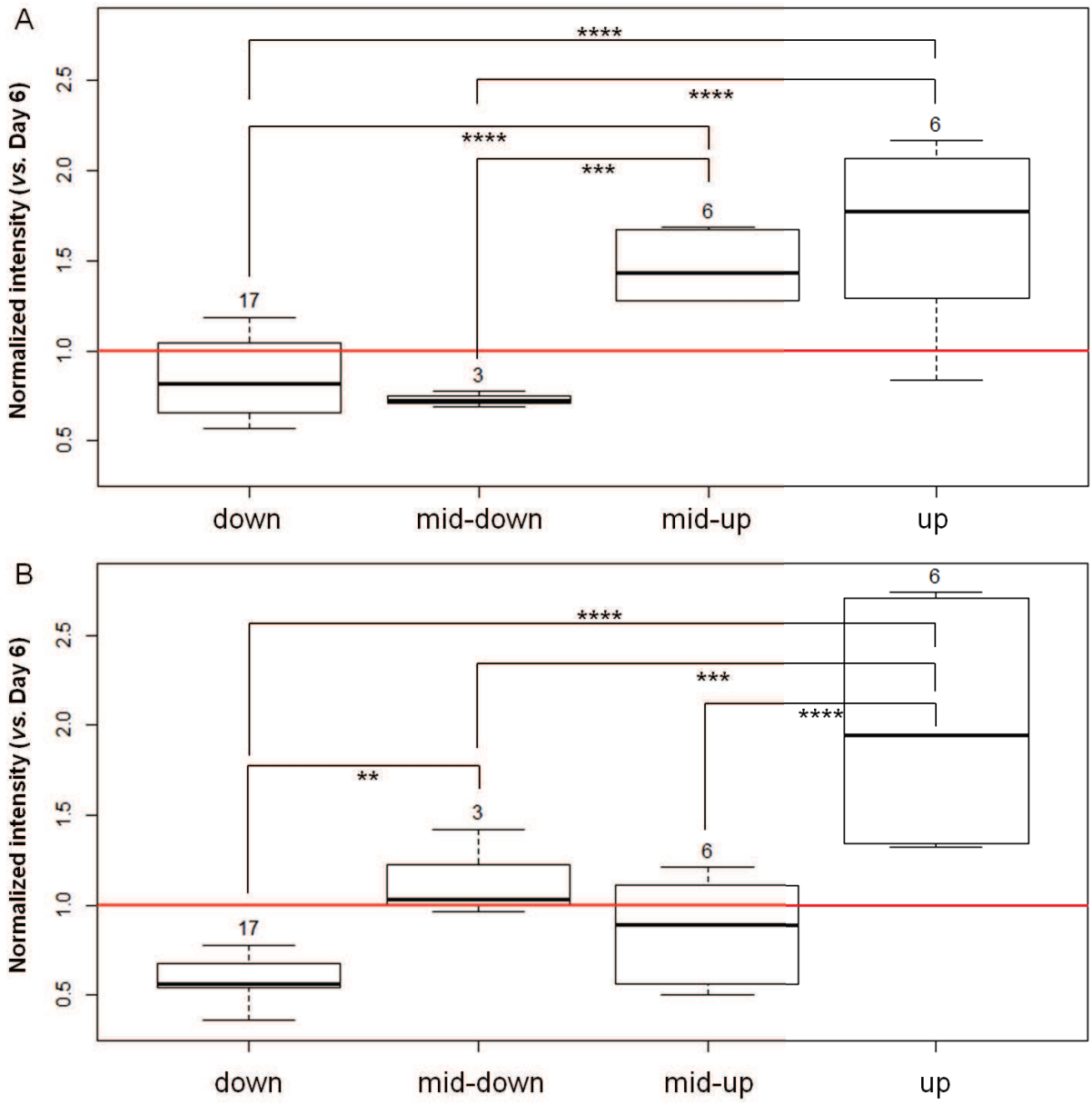


Figure 16: Comparison of mean spot intensities as a function of spot evolution category. Boxplots represent the mean normalized spot intensities of each of the four categories of spot evolution, at day 27 (A) and at day 41 (B). The red horizontal line represents the reference i.e. spot intensity at day 6. Numbers on top of each boxplot are the population size. Stars represent the degree of significance of the difference between the groups, calculated by ANOVA and TukeyHSD: ** $p < 0.05$, *** $p < 0.01$ and **** $p < 0.001$.

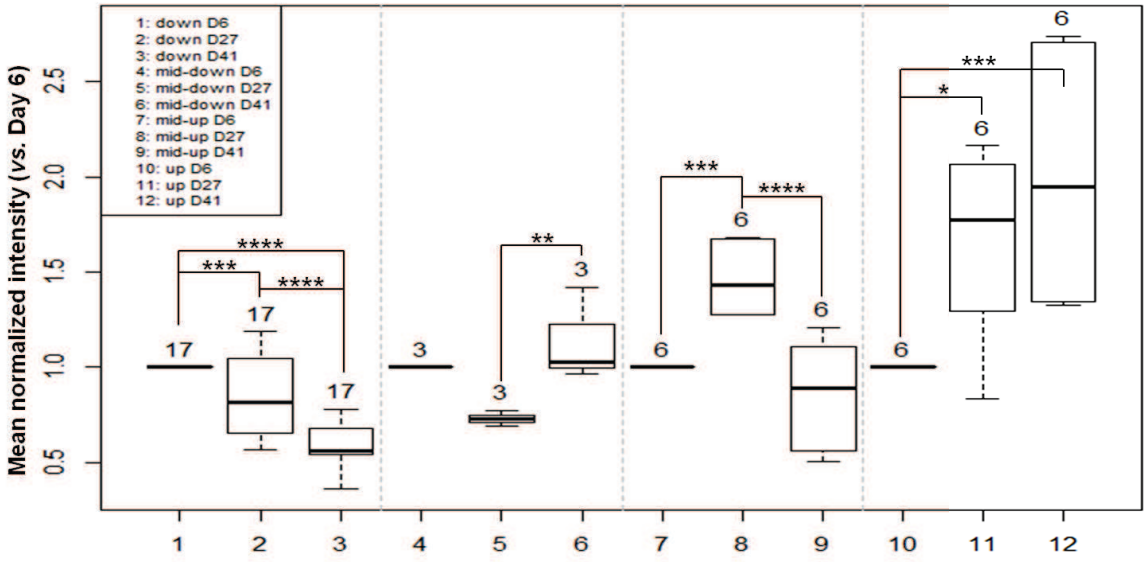


Figure 17: Comparison of mean spot intensities as a function of storage duration. Boxplots represent the mean normalized spot intensities of each of the four categories of spot evolution at days 27 and 41. Numbers on top of each boxplot are the population size. Stars represent the degree of significance of the difference between the two time-points of each category, calculated by Student paired t-test: * $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$ and **** $p < 0.001$.

Seventeen spots showed a mean decreasing tendency, either decreasing between days 6 and 27, then being stable until day 41 (9 spots), or being stable between days 6 and 27, then decreasing until day 41 (6 spots). Additionally, 2 spots tended to decrease continuously throughout the storage period. The decreasing intensity means that less reversible cysteine oxidation is detected, either explained by cysteine residues becoming free over time or on the contrary by the appearance of irreversible oxidative states, making the labeling process inefficient, which is the case for the sulfonic acid R-SO_3 state. The decrease can also be explained by the disappearance of oxidized proteins by proteolysis or expulsion out of the cell.

Six spots appeared to increase during the storage period, mostly between days 6 and 27 (5 spots). Only one spot was stable between days 6 and 27, and then increased during the two last weeks of the storage period. The increase in intensity over time can only be explained by an accumulation of reversibly oxidized copied of proteins. Some other spots (9) presented a peak pattern at day 27, either a top peak (6 spots) or a down peak (3). The top-peak pattern can be

due to a starting accumulation of reversible oxidation further progressing to irreversible states of oxidation. The down-peak pattern could correspond to a specific oxidized protein population being efficiently degraded in the beginning of the storage period. Afterwards, for some unknown reason, the degradation could be altered, resulting to the accumulation of these oxidized proteins.

Once the data processing for spot segregation into the four categories validated, it was possible to investigate the effect of storage duration on each category, as depicted on Figure 17. One-way ANOVA revealed really significant effect of time of storage on spots presenting a mean decreasing pattern ($p < 5 \cdot 10^{-10}$) or a mean increasing pattern ($p < 0.005$). As for spots presenting a mid-peak pattern, mean normalized intensity at day 41 comes back to around 1, so the storage duration do not appear to have an effect in respect with the three storage durations investigated ($p = 0.6388$ and $p = 0.7317$ for the mid-down and mid-up categories, respectively). Considering all the 32 spots of interest, there was no statistically significant global effect of storage duration on spot intensity ($p = 0.8383$). However, the pairwise multiple comparisons revealed significant differences between some couples of storage durations, even in the categories of spots with a peak pattern (see details on Figure 17). This means that proteins are differentially altered by oxidative processes at their cysteine residues during the storage period. Several oxidative states are involved, with more or less deleterious effect, and it is very probable that some protein populations, thus some molecular functions are specifically affected through cysteine oxidation.

In addition to its major implications in protein structure and function and in cellular processes, we have shown here that the oxidation of cysteine residues also occurs as a marker of RBCs aging during their storage. Moreover, it seems that this oxidative hallmark of RBCs storage is not a random phenomenon since we successfully highlighted different categories of evolution of this modification over the storage period. As a consequence, it is likely that specific populations of proteins are affected, which will be investigated in Part 3 through the MS-based identification of proteins present in the detected spots of interest.

Chapter two

Evolution of protein carbonylation

This chapter refers to the article

"Subcellular fractionation of stored red blood cells reveals a compartment-based protein carbonylation evolution"

Published in

Journal of Proteomics, 76 (2012) 181-193

Introduction

Protein carbonylation is also commonly investigated as a marker of oxidative stress [31, 32] and aging [33-35] in many eukaryotic cell populations, including RBCs [36-38]. This irreversible modification consists in the introduction of aldehyde or ketone chemical functionalities onto amino acid side chains (mainly at arginine, lysine, proline and threonine residues), or results from protein backbone cleavage through α-amidation or β-scission [39]. Contrary to cysteine oxidation, protein carbonylation does not target a specific amino acid involved in cellular function such as the redox regulation of the cell. However, the multiplicity of potential targeted amino acids, thus the multiple changes that a protein can undergo through carbonylation, makes higher the probability to affect active sites (for instance, see a non-exhaustive list of amino acid carbonylation products in reference [40]). Amino acid carbonylation can directly affect an active site or impact the protein structure, both affecting protein function.

Several approaches were developed to assess protein carbonylation [41, 42]. Basically, there are two main existing strategies to detect and quantify this modification. They are based either on reaction with tritiated borohydride, or on derivatization with 2,4-dinitrophenylhydrazine (2,4-DNPH). The former relies on borohydride-induced reduction of carbonyl groups to alcohols, with incorporation of a tritium atom, and radioactivity determination by scintillation counting. The latter is based on a condensation reaction (loss of water) that leads to the formation of a protein-hydrazone complex, whose optical density allows quantitation of carbonylation. If the tritiated borohydride approach appears to be more sensitive as a test for aldehydes or ketones detection, the method based on 2,4-DNPH derivatization presents the advantage of the availability of a wider range of commercial reagents, and easiness of derivatization detection by photometry, compared to radioactivity. Moreover, more hindsight are available relative to this approach, through literature from influent research groups, renowned in the fields of transfusion medicine and protein chemistry (groups of Professor Zolla (Tuscia university, Viterbo, Italy), Professor Papassideri (University of Athens, Athens, Greece), and Professor Regnier (Purdue University, IN, USA).

Several methods based on derivatization with hydrazide-containing compounds have thus been

developed for detection and quantitation of carbonyl protein content [43-48]. Among those utilizing 2,4-DNPH derivatization, the apparently simplest one is a spectrophotometric assay. Indeed, the resulting protein-hydrazone complexes absorb in the UV at 375 nm, with a molar extinction coefficient of 22000 $M^{-1} \cdot cm^{-1}$. Erythrocyte samples first need to be depleted in Hb since this extremely abundant RBC protein also absorbs at this wavelength. Moreover, a protein precipitation step is required following the derivatization reaction in order to get rid of excess of derivatizating reagent that otherwise will interfere with the absorbance reading. The availability of antibodies directed against DNP moieties made possible to perform carbonylation quantitation through western-blotting or ELISA assays. Compared to photometric assay, these approaches present the advantage of running several samples in a same analysis, limiting the experiment-related variability. Moreover, there is no technical need for Hb depletion since the detection is immunologically-mediated. As for the western-blot approach, reagent removal after derivatization is unnecessary because excess of 2,4-DNPH is removed during SDS-PAGE. There is thus no need for precipitation step, avoiding potential protein loss. In the same manner, low molecular-weight non-protein compounds such as lipids or sugars that can bear carbonyl groups will migrate out of the gel and will not interfere for the quantitation of protein carbonylation. Finally, the gel-based experiments can give access to an additional kind of information, the molecular weight (MW) of carbonylated proteins.

Such approach has allowed investigations on protein carbonylation in stored RBCs, bringing Papassideri's [36, 37] and Zolla's [49] groups to conclude to an increase in carbonylated protein content. In particular, Kriebardis *et al.* have shown via immunodetection of 2,4-DNPH derivatized proteins that the oxidative index of non-leukodepleted CPDA-stored (CDP anticoagulant plus adenine) erythrocyte membrane proteins increased along the storage period [36], later emphasized to the cytoskeletal proteins [37]. Moreover, Zolla *et al.* have shown that total erythrocyte protein carbonylation increased uniformly during the first four weeks of storage in leukodepleted, CPDA-collected, SAGM-stored RBCs, followed by a steady state evolution or slight decrease [49]. They have also measured an accumulation of ROS and lipid oxidation increasing parallel to protein carbonylation. Metabolic investigations allowed them to correlate these findings with an over-activation of the oxidative phase of the pentose

phosphate pathway, leading to the conclusion that protein carbonylation is due to an increasing oxidative stress along storage, reaching a maximum level after three weeks [49].

Study design

The first step of this work was to develop a subcellular fractionation in order to investigate the evolution of protein carbonylation on the four following protein populations: soluble Hb fraction, Hb-depleted soluble fraction, integral protein-enriched membrane fraction and cytoskeleton-enriched membrane fraction. Then, three independent ECs (namely EC6, EC7 and EC8) were followed weekly (see donor details in annex A). Cell lysis, separation of membranes from soluble fraction, and membranes washes were performed on the collection day before sample freezing. Further fractionation processes were performed just before protein carbonylation analyses, once all samples were collected. The study design is pictured out on Figure 18.

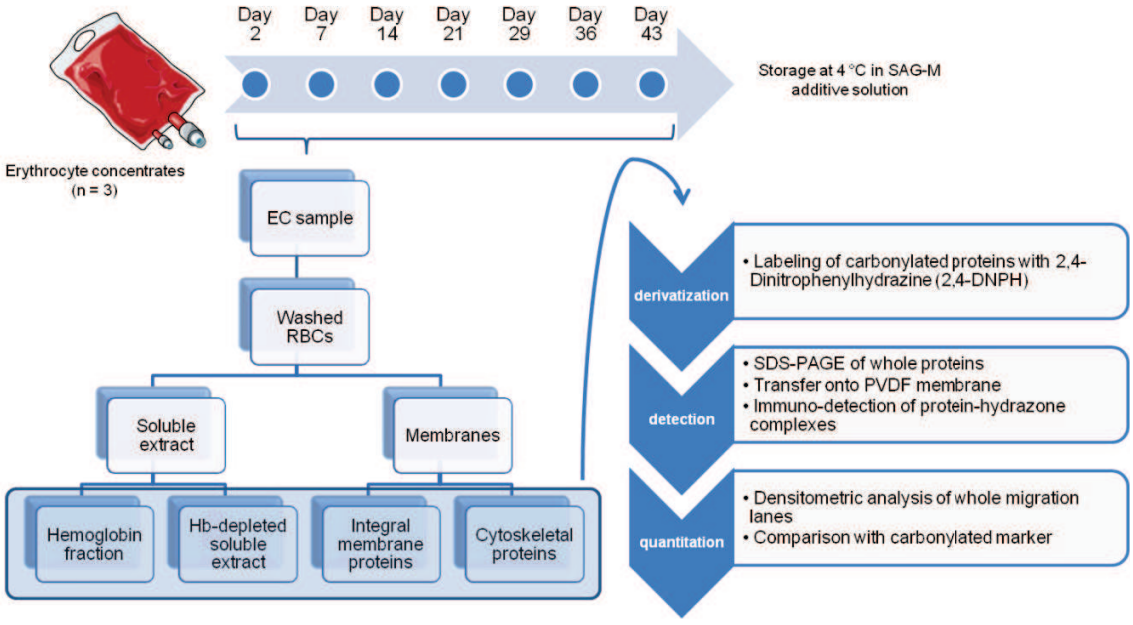


Figure 18: Study design for the quantitative evolution of protein carbonylation in subcellular fractions of stored RBCs. Weekly collected samples were fractionated into four subcellular extracts (soluble Hb fraction, Hb-depleted soluble extract, integral protein membrane and cytoskeleton membrane DDM and DC extracts), then carbonylated proteins were derivatized with 2,4-DNPH, and detected by western-blot. Densitometric analyses allowed the quantitation of carbonylation in the four protein populations over the storage of ECs.

Material and methods

Collection and subcellular fractionation of erythrocytes

Erythrocytes were collected weekly at days 2, 5*, 7, 14, 21, 29, 36 and 43 (*: note that for EC6, a sample was taken at day 5 instead of days 2 and 7, due to a provision delay of the concentrate after its rejection from the blood bank). Erythrocytes were first spun down at 2000 *g* during 10 minutes at 4 °C. RBCs were then washed three times in a physiological 0.9% NaCl solution and spun down with the same conditions as before, to get them free of additive solution and hemolysis products. RBCs were lyzed by one-hour incubation at 4 °C in a hypotonic 0.1x PBS solution under agitation. Then, cytoplasmic extracts and membranes were separated by centrifugation at 18000 *g* during 30 minutes at 4 °C.

Hb was depleted from the soluble cytoplasmic extracts by nickel-based IMAC chromatography with a HisTrap HP 5 mL column (GE Healthcare, Fairfield, CT, USA) on a BioLogic Workstation (BIO-RAD, Hercules, CA, USA), based on ref [29]. The flow through (RBC soluble Hb-depleted fraction) was collected in a single fraction, and the corresponding Hb fraction was eluted with a gradient of imidazole-containing PBS buffer, from 10 to 100 mM in 20 minutes.

RBC membranes were washed several times with the lysis solution until no more soluble Hb was released in the wash supernatant after 30 minutes of centrifugation at 18000 *g* at 4 °C. Typically, membranes free of soluble Hb were obtained after 3 washes in a buffer-to-membrane volume ratio of 2, followed by 4 washes in a buffer-to-membrane volume ratio of 10. Washed membranes were then sequentially solubilized with 5 volumes of dodecyl-maltoside (DDM) buffer composed of 7.8 mM DDM in 50 mM Tris-HCl, 750 mM aminocaproic acid, 0.5 mM EDTA, pH 7.0, vortexed and centrifuged (same conditions as before), which gave the first membrane protein extract (DDM membrane fraction). DDM is a non-ionic detergent allowing extraction of integral membrane proteins (based on ref [50]). Afterwards the remaining pellet was solubilized with 5 initial membrane volumes of deoxycholate (DC) buffer, composed of 1 % DC in 50 mM Tris-HCl, 150 mM NaCl, pH 8.1, and vortexed. DC is an anionic detergent allowing to solubilize the DDM-insoluble material, *i.e.*peripheral membrane proteins (based on ref [51]). After a last centrifugation (same conditions as before), the supernatant contained the second membrane

protein extract (DC membrane fraction). It has to be noted that no wash was performed between DDM and DC extractions in order to keep all the carbonylated membrane proteins.

Protein assay and sample preparation

Protein concentrations of RBC Hb-depleted fraction and membrane extracts were determined using the Bradford Protein Assay (BIO-RAD, USA), with a calibration curve composed of BSA dilutions. The concentration of Hb fractions was calculated thanks to the Harboe direct spectrophotometric method [52] with the 3-point "Allen correction" [53]. Harboe's method has the advantage to be the more accurate Hb measurement method in the presence of interfering substances [54]. Hemoglobin-depleted soluble extracts were concentrated via centrifugation at 4750 *g* through Vivaspin ultrafiltration spin columns (Sartorius, Götingen, Germany) with a 5000 Da MW cut off. Lower-volume extracts (DDM and DC membrane extracts, both 500 µL) were vacuum centrifuged upon dryness and resuspended both in DC buffer to desired concentration.

Detection of carbonylated proteins

Protein carbonylation content was followed along the storage of three ECs as mentioned earlier. Carbonylation detection and quantitation experiments were performed with the Oxyblot™ Protein Oxidation Detection Kit (Millipore, Billerica, MA, USA), with 15 to 20 µg of proteins. Proteins were derivatized with 2,4-DNPH according to the manufacturer's instructions. Briefly, samples were mixed with SDS for protein denaturation, and incubated for 15 minutes at room temperature with the 2,4-DNPH derivatization solution. A RBC soluble extract was oxidized via a metal-catalyzed oxidation reaction and was used as derivatization positive control (through incubation with the derivatization solution) as well as negative control (through incubation with the control derivatization solution that did not contain 2,4-DNPH). The derivatization reaction was then stabilized by addition of the neutralization solution, and samples were finally reduced with β-mercaptoethanol (final concentration 0.74 M). SDS-PAGE protein separation was then performed using NuPAGE® 4-12 % Bis-Tris precast mini-gels (Invitrogen, USA), and gels were run individually in MOPS buffer (Invitrogen, USA) with a constant low voltage of 100 V. Proteins were then transferred onto Hybond™ hydrophobic polyvinylidene difluoride (PVDF) membranes (GE Healthcare, USA) using a liquid transfer system. Transfer efficiency as well as homogenous protein quantity loading were checked with membrane Ponceau Red staining. Membranes were

then blocked overnight at 4 °C by incubation with a 5 % BSA, 0.1 % Tween 20, in PBS solution under agitation. Immunodetection of carbonylated proteins was performed by incubating membranes with a primary antibody solution (rabbit anti-DNP IgG antibody) during 1h at RT, followed by three washes with 0.1 % Tween 20 in PBS solution. Afterwards membranes were incubated with a secondary antibody solution (HRP-conjugated goat anti-rabbit IgG antibody) during 1h at RT, and then washed three times as before. Carbonylated proteins were revealed by Enhanced Chemiluminescence (ECL™ Western Blotting Detection Reagents, GE Healthcare, USA) reaction. Films (Amersham Hyperfilm™ ECL, GE Healthcare, USA) were then scanned with a Personal Densitometer SI (GE Healthcare, USA), and quantitation of protein carbonylation content was achieved with the ImageQuant TL 7.0 software (GE Healthcare, USA).

Quantitation of protein carbonylation per EC

The quantitation of carbonyl groups was performed by densitometric analysis of the immunodetection films. Quantitation was assessed on whole migration lanes for each sample in order to work with the entire carbonylation status of each RBC fraction. Densitometric values were measured and expressed by the software as “volumes”, then converted in femtomoles of carbonyl groups by comparison with the densitometric volume of the carbonylated BSA, included in the Oxyblot mixture of standard proteins (band at 68 kDa), and of which the carbonylation status is known. According to the quantity of proteins derivatized and loaded for SDS-PAGE, the quantitation was then reported as femtomoles of carbonyl groups per microgram of proteins (fmol/µg). Finally, considering extract protein concentrations and fraction volumes, results were expressed as µmol/EC.

Statistics

Reproducibility of the quantitation of protein carbonylation was evaluated on triplicates of measures. The mean CV % was determined as 7.2 %.

Statistics were evaluated using the SigmaStat 3.10 software (SYSTAT Software Inc., Point Richmond, CA, USA). Significant evolution of carbonylation during storage was determined using the one-way Analysis of Variance (ANOVA) followed by a Tukey test for pairwise multiple comparisons. As for EC6, data at day 7 were interpolated via a linear regression between values

at days 5 and 14. Differences were considered statistically significant for p-values < 0.05.

Results and discussion

Protein carbonylation in stored RBCs

A sequential extraction of membrane proteins was set up to enable the separation of integral membrane proteins from cytoskeletal ones, beyond the main membrane/cytosol cellular fractionation. The characterization of both membrane extracts is described in annex B. The Carbonylated proteins were detected by anti-DNP immunodetection after 2,4-DNPH derivatization of protein extracts from each of the four RBC subcellular fractions mentioned above (*i.e.* soluble Hb fraction, Hb-depleted soluble fraction, DDM membrane fraction and DC membrane fraction). Protein carbonylation appeared to be a specific modification affecting proteins in a non-random manner. In particular, Figure 19 shows two proteins with similar Ponceau red staining intensities (total protein detection), but different immunodetection intensities (specific carbonylated proteins), proving that carbonylation quantitation is not determined by protein abundances.

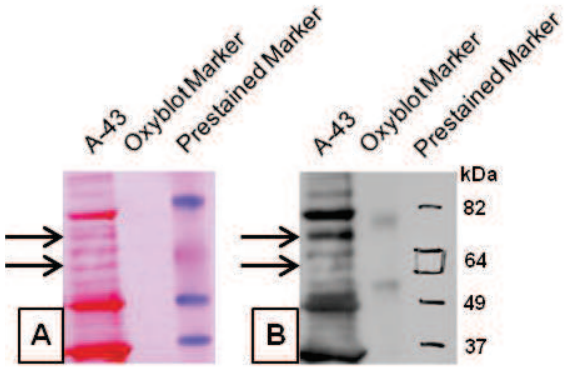


Figure 19: Specificity of protein carbonylation modification. A: Ponceau red staining of a part of a PVDF membrane onto which a sample from Hb-depleted soluble fraction (EC6, day 43) was transferred, as well as a carbonylated protein ladder ("Oxyblot Marker") and a prestained protein ladder ("Prestained Marker"). B: Film corresponding to the same part of the membrane after the immunodetection of DNP moiety-containing proteins (i.e. derivatized carbonylated proteins). The arrows show two proteins with similar Ponceau red staining intensities, but different immunodetection intensities, proving that the carbonylation is a specific modification that does not affect proteins according to their abundance.

Carbonylation in RBC soluble fractions

The quantitation of carbonylated protein contents in soluble Hb and in Hb-depleted soluble

fractions during the storage of RBCs revealed that Hb carbonylation level varied between 40 and 90 fmol/µg (Figure 20A) whereas the one of the Hb-depleted soluble fraction was comprised between 300 and 1000 fmol/µg (Figure 20B). Soluble Hb is thus around 10 times less carbonylated than the pool of all the other soluble proteins. However, Hb is much more abundant than other proteins in RBC cytoplasm. Indeed, it represents 96.96 ± 0.25 % of the RBC soluble protein content (measured from 18 soluble extracts). When dealing with the entire EC, between 1 and 4 µmol of total carbonyl groups were carried by soluble Hb (Figure 21A) and between 0.2 and 1.7 µmol were carried by all the other soluble proteins (Figure 21B). The mean ratio between the carbonylation carried by soluble Hb and the carbonylation carried by the rest of soluble proteins along the storage of the three ECs monitored during storage is 3.74 ± 1.51. This value is coherent with a global ratio of 3.19, which is the combination of the ratios of protein abundance (96.96/3.04 = 31.89 in favor of Hb) and carbonylation status (10 in favor of the rest of soluble proteins).

Soluble Hb and Hb-depleted soluble fractions present both similar carbonylation patterns along the storage. As a result, it appeared that the total soluble carbonylation of each EC monitored presented a decrease of 23.1 %, 26.1 % and 36.6 % (EC6, EC7 and EC8, respectively) in the beginning of the storage period afterwards it was globally stable (Figure 21C).

Carbonylation in RBC membrane fractions

Both DDM and DC membrane extracts showed protein carbonylation rates of the same order of magnitude than the Hb-depleted soluble fraction one, *i.e.* a few hundreds of fmol/µg all along the storage period. However, DDM fraction protein carbonylation rate was a little bit lower (300 to 500 fmol/µg) than the DC fraction one (500 to 1000 fmol/µg) (Figure 20C and D). This approximately two-fold difference was conserved in terms of membrane carbonylation content in ECs, which fluctuated between 0.4 and 1.2 µmol/EC for DDM extracts and between 0.5 and 2.5 µmol/EC for DC extracts (Figure 21D and E). Carbonylation content along storage showed interesting patterns in both membrane extracts. Concerning DDM extracts, a constant level of carbonylation was observed until day 21. These mean basal values of 0.400, 0.445 and 0.732 µmol for EC6, EC7 and EC8 respectively, were taken as 100 % of DDM fraction carbonylation for each corresponding EC. Then, for two of the three ECs followed (EC7 and EC8), an increase of 51

% and 64.5 % was observed between days 21 and 29, respectively. Finally, carbonylation fell down to less than its basal level (93.5 % and 73.8 %, respectively) until the end of the storage period. The third EC followed (EC6) showed a constant carbonylation level at around 0.4 µmol/EC throughout the storage period (Figure 21D).

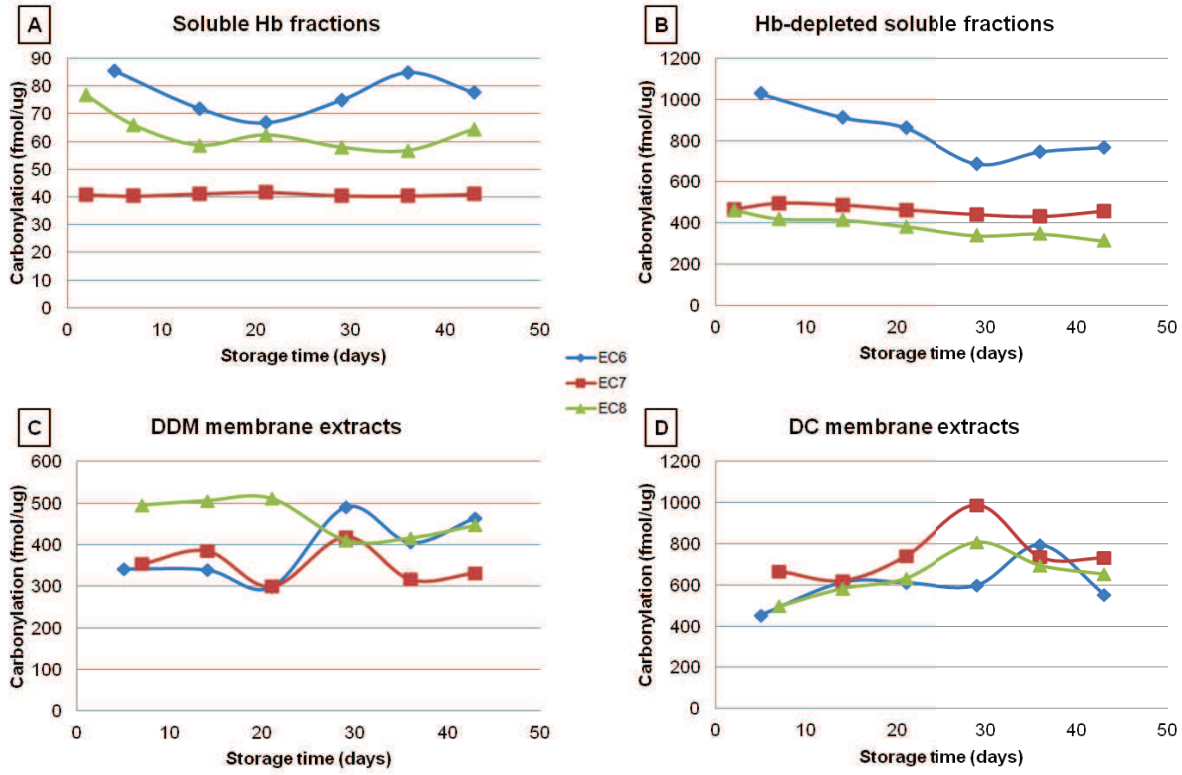


Figure 20: Protein carbonylation rates in subcellular extracts. Protein carbonylation rates along the storage in Hb (A) and Hb-depleted (B) soluble extracts, and in DDM (C) and DC (D) membrane extracts. In terms of quantity of carbonylation per quantity of protein, data in soluble extracts are globally stable throughout the storage period whereas there is a slight global increase of protein carbonylation rates in the two membrane extracts.

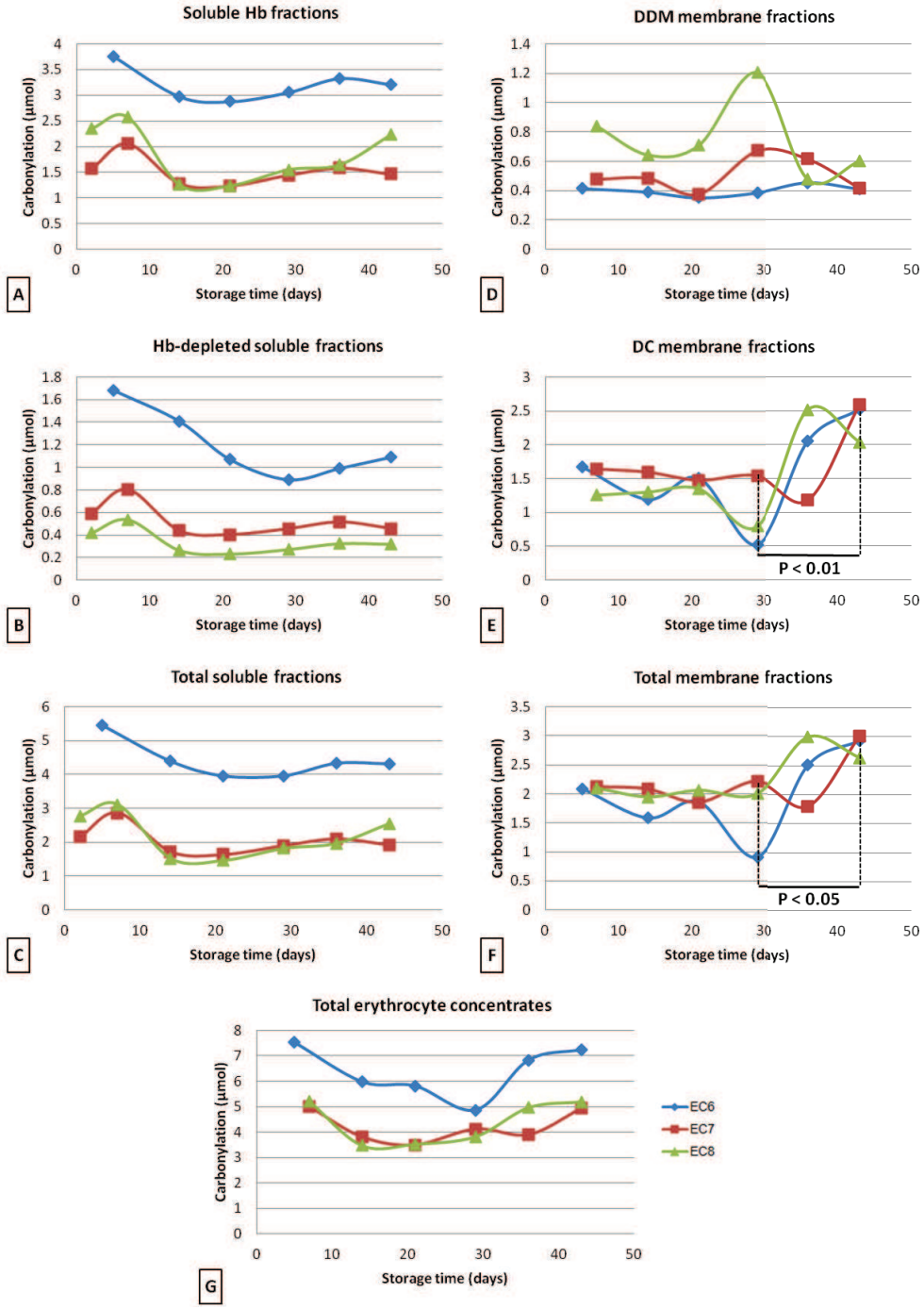


Figure 21: Absolute quantitation of protein carbonylation along the storage period of ECs. Quantitation of carbonylated proteins in ECs in Hb (A), Hb-depleted (B) and total (C) soluble fractions, in DDM (D), DC (E) and total (F) membrane fractions, and in total erythrocyte concentrates (G). The carbonylation content of total soluble and total membrane fractions is the sum of Hb and Hb-depleted soluble fractions, and of DDM and DC membrane fractions, respectively. Soluble Hb carbonylation accounts for half of the total carbonylation, thus attenuating the influence of the other compartments. Membrane fraction carbonylation is however of interest due to its fluctuating patterns, and tends to increase in the end of storage. The increase of carbonylation between days 29 and 43 is statistically significant in DC membrane fractions ($p < 0.01$) and in total membrane fractions ($p < 0.05$).

DC membrane extracts also revealed an increasing pattern of carbonylation content, but in a different way. Indeed, the carbonylation content was also stable at 1.5 and 1.3 µmol from day 7 to day 21 for EC6 and EC8, and at 1.6 µmol from day 7 to day 29 concerning EC7. These mean basal values were taken as 100 % of DC fraction carbonylation for each corresponding EC. Then, EC6 and EC8 presented a decrease of 64.0 % and 38.8 %, respectively, until day 29, and EC7 presented a decrease of 24.7 % until day 36. Finally, the carbonylation content reached up to 173.0 %, 165.5 % and 174.9 % until the end of the storage period for EC6, EC7 and EC8 respectively (Figure 21E). Effect of storage on protein carbonylation evolution in DC membrane extracts was found statistically significant ($p \cong 0.01$), with a particular significant increase between days 29 and 43 ($p < 0.01$).

The global pattern of RBC membrane carbonylation (Figure 21F) appeared stable until day 29 at around 2 µmol for EC7 and EC8, and then increased up to 3 µmol until the end of the storage period. As for EC6, the global membrane pattern presented the same decrease after day 29 than in the DC fraction, due to the stable carbonylation of DDM fraction proteins in this EC, as shown in Figure 21D. Effect of storage on the membrane protein carbonylation was statistically significant ($p < 0.05$), as well as the carbonylation increase between days 29 and 43 ($p < 0.05$).

Carbonylation in whole RBCs

Adding the carbonylation content of each RBC compartment, this storage lesion may appear stable in the entire RBCs since similar amounts were calculated at day 7 and at day 43 (around 7.3 µmol for EC6 and 5.0 µmol for EC7 and EC8, Figure 21G). However, this global pattern was divided in a starting soluble-related decrease and an ending membrane-related increase of carbonylation, as described above. These variations are more easily observed in Figure 22 where one can see, expressed in percentages, the means distribution of protein carbonylation and total protein content throughout the storage period (Figure 22A-1 to A-6, and Figure 22B-1 to B-6, respectively). Moreover, the part of carbonylation content in each fraction highly differed from the protein abundance repartition (Figure 22A *vs.* Figure 22B), again highlighting the non-random occurrence of protein carbonylation.

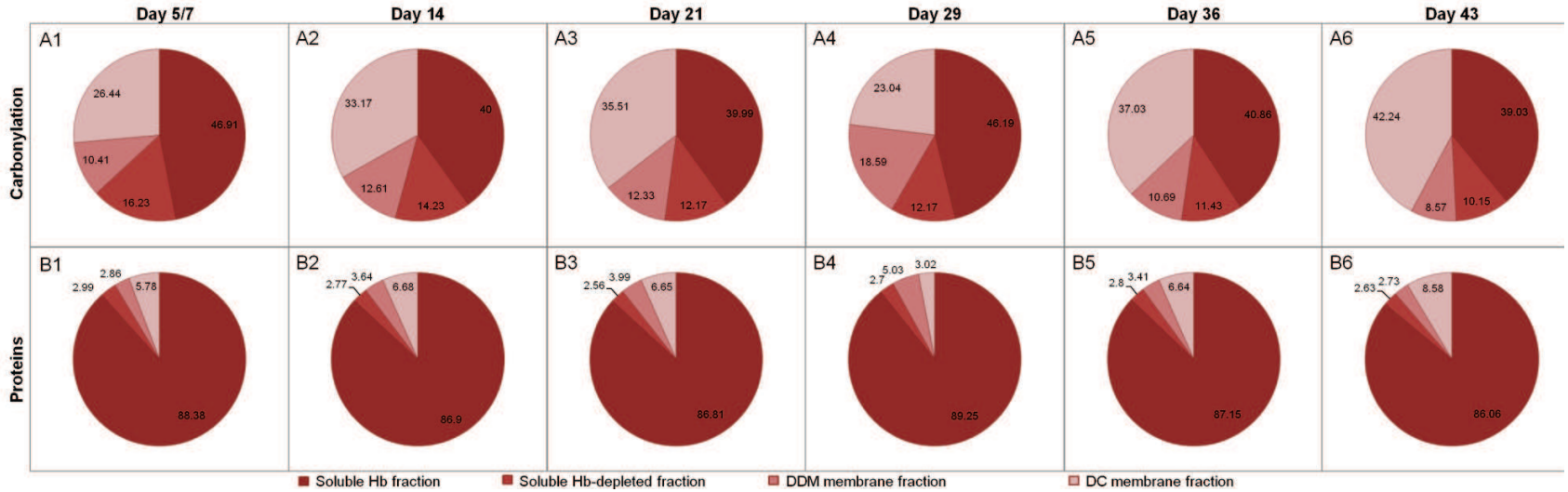


Figure 22: Mean repartition of protein carbonylation and total protein content in RBC compartments during storage. The proportions of carbonylation and protein content in each fraction are shown according to storage duration (A1 to A6 and B1 to B6, respectively). Data are the means of the three ECs followed. One can notice that the carbonylation distribution in each compartment does not match the protein repartition, indicating a targeted modification. Carbonylation in DC membrane fraction increases remarkably during the second half of storage.

Conclusions

First of all, the sample fractionation has become a nearly mandatory step in every proteomic analysis. The dynamic range improvement is a very important parameter to access the more comprehensive information possible. Hence, the protein oxidation (both cysteine oxidation and protein carbonylation) was explored in soluble and/or membrane compartments. Hb being the ultra-abundant erythrocyte soluble protein, it was successfully depleted from the cytoplasmic fraction, allowing a better investigation of its whole pool of proteins. Such depletion procedure is particularly important in gel-based approaches where data rely on visual information. Global membrane content was also fractionated since some proteins such as spectrin chains and band 3 are also very abundant. For instance Burton and Bruce highlighted the high protein abundance range in red cell membranes with extremes being band 3 and CR1 ($1.2 \cdot 10^6$ *vs.* 20 copies per cell, respectively) [55]. A sequential membrane protein extraction was thus established for the protein carbonylation investigation, allowing the separation of integral membrane and cytoskeletal proteins.

Protein oxidation during the EC storage of RBCs was here investigated as both transient and irreversible oxidative states, through the detection of proteins bearing reversibly oxidized cysteine residues or irreversible acquisition of reactive carbonyl functional groups, respectively. In a first part, a redox-DIGE approach allowed to detect reversibly oxidized proteins in stored erythrocytes, and to study their evolution over the storage period. Up to date, and to the best of our knowledge, this is the first time that such a study is performed in a transfusion medicine-related point of view. Studying the evolution of protein cysteine oxidation throughout the storage of blood products, and in particular of ECs, is of great interest since oxidation-reduction of reactive thiol species plays an important role in health and disease biological processes. *In vitro* storage of banked erythrocytes is often associated with their *in vivo* aging, for which the oxidative challenge undergone by RBCs seems to be of importance. This study brings new quantitative data related to protein oxidation. It can be concluded that cysteines of proteins from Hb-depleted soluble extracts of stored RBCs are not equally affected by oxidation. Beyond

the kind of modification (disulfide, glutathionylation, nitrosylation, sulfenylation and so on), the oxidation evolution along the storage duration differs among affected proteins. The different categories of evolution allowed us to determine groups of proteins either being more and more oxidized until moving to an irreversible state, or staying in a reversible state, or proteins being less oxidized throughout the storage, suggesting an action of antioxidant enzymes or a degradation of such proteins. Cysteine oxidation thus appears as a heterogeneous modification targeting specific proteins.

As for the carbonylation of proteins, several studies have already characterized its extent over the storage period. Through this study, we obtained data partially in adequacy with the existing literature. Indeed, we were able to highlight end-storage related significant changes in membrane and associated skeleton only after three to four weeks of storage, whereas others detected changes right after two weeks. In particular, this storage limit of 14 days, proposed by Koch based on debatable poorer post-surgery transfusion-related clinical outcomes [56], seems to fit with the work conducted by Zolla and coworkers [49]. In addition to confirm a time-related evolution of the protein carbonylation content, this study also highlighted a particularly interesting subcellular compartment-dependent evolution of protein carbonylation. In blood flow as well as in ECs, a heterogeneous population of RBCs is encountered in term of cell age (up to 120 days *in vivo*). A mean basal carbonylation content is thus quantified early in the storage period. Thanks to data obtained, it is now clear that RBCs undergo changes in protein carbonylation content along the storage period, and that these changes are compartment-dependent. During the first two weeks of storage, a decrease of the basal carbonylation was quantified in soluble fractions. RBCs are known to be well equipped in antioxidant defenses, preventing or slowing down effects of oxidative stress. Moreover, proteasomal activity has been described in human RBCs [57], with an aspecific affinity for mildly oxidized proteins [58]. Such activity could explain the loss of protein carbonylation observed in soluble fractions. As for membrane extracts, protein carbonylation showed a significant increase from three to four weeks of storage. This increase is however attenuated with a decrease of carbonylation in integral membrane fractions at the end of the storage period. It is of particular interest to note that throughout this period, RBCs have been shown to release an exponential amount of

microvesicles [59] enriched in membrane proteins [60, 61]. This can possibly explain the dramatic loss in membrane protein carbonylation in the end of the storage.

Both cysteine oxidation and protein carbonylation are modifications able to alter protein functions. The big deal of this redox investigation is now to put names on the altered proteins. Two possibilities are to be considered here: either oxidative lesions globally affect all types of RBC proteins, or some protein populations, thus some protein functions, are targeted by a specific mechanism. In order to answer this question, we chose to apply existing and newly-develop mass-spectrometry based approaches for identification of cysteine-oxidized and carbonylated proteins, respectively. This part of my research will be explored hereafter.

References

1. Maizels, M. and J.H. Paterson, *Survival of stored blood after transfusion.* The Lancet, 1940. **236**(6110): p. 417-420.
2. Gabrio, B.W., A.R. Stevens, Jr., and C.A. Finch, *Erythrocyte preservation. III. The reversibility of the storage lesion 1.* The Journal of Clinical Investigation, 1954. **33**(2): p. 252-256.
3. Biteau, B., J. Labarre, and M.B. Toledano, *ATP-dependent reduction of cysteine-sulphinic acid by S. cerevisiae sulphiredoxin.* Nature, 2003. **425**(6961): p. 980-4.
4. Chang, T.S., et al., *Characterization of mammalian sulfiredoxin and its reactivation of hyperoxidized peroxiredoxin through reduction of cysteine sulfinic acid in the active site to cysteine.* J Biol Chem, 2004. **279**(49): p. 50994-1001.
5. Sommer, A. and R.R. Traut, *Diagonal polyacrylamide-dodecyl sulfate gel electrophoresis for the identification of ribosomal proteins crosslinked with methyl-4-mercaptobutyrimidate.* Proc Natl Acad Sci U S A, 1974. **71**(10): p. 3946-50.
6. Brennan, J.P., et al., *Detection and mapping of widespread intermolecular protein disulfide formation during cardiac oxidative stress using proteomics with diagonal electrophoresis.* J Biol Chem, 2004. **279**(40): p. 41352-60.
7. Henriksen, U., et al., *Identification of intra- and intermolecular disulfide bridges in the multidrug resistance transporter ABCG2.* J Biol Chem, 2005. **280**(44): p. 36926-34.
8. Baty, J.W., M.B. Hampton, and C.C. Winterbourn, *Detection of oxidant sensitive thiol proteins by fluorescence labeling and two-dimensional electrophoresis.* Proteomics, 2002. **2**(9): p. 1261-6.
9. Leichert, L.I. and U. Jakob, *Protein thiol modifications visualized in vivo.* PLoS Biol, 2004. **2**(11): p. e333.
10. Brennan, J.P., et al., *The utility of N,N-biotinyl glutathione disulfide in the study of protein S-glutathiolation.* Mol Cell Proteomics, 2006. **5**(2): p. 215-25.
11. Jaffrey, S.R., et al., *Protein S-nitrosylation: a physiological signal for neuronal nitric oxide.* Nat Cell Biol, 2001. **3**(2): p. 193-7.
12. Saurin, A.T., et al., *Widespread sulfenic acid formation in tissues in response to hydrogen peroxide.* Proc Natl Acad Sci U S A, 2004. **101**(52): p. 17982-7.
13. Jaffrey, S.R. and S.H. Snyder, *The biotin switch method for the detection of S-nitrosylated proteins.* Sci STKE, 2001. **2001**(86): p. pl1.
14. Unlu, M., M.E. Morgan, and J.S. Minden, *Difference gel electrophoresis: a single gel method for detecting changes in protein extracts.* Electrophoresis, 1997. **18**(11): p. 2071-7.
15. Fu, C., et al., *Quantitative analysis of redox-sensitive proteome with DIGE and ICAT.* J Proteome Res, 2008. **7**(9): p. 3789-802.
16. Riederer, I.M., et al., *Serial protein labeling with infrared maleimide dyes to identify cysteine modifications.* J Proteomics, 2008. **71**(2): p. 222-30.
17. Izquierdo-Alvarez, A. and A. Martinez-Ruiz, *Thiol redox proteomics seen with fluorescent eyes: the detection of cysteine oxidative modifications by fluorescence derivatization and 2-DE.* J Proteomics, 2011. **75**(2): p. 329-38.
18. Klatt, P. and S. Lamas, *Regulation of protein function by S-glutathiolation in response to oxidative and nitrosative stress.* Eur J Biochem, 2000. **267**(16): p. 4928-44.

19. Giustarini, D., et al., *S-glutathionylation: from redox regulation of protein functions to human diseases.* J Cell Mol Med, 2004. **8**(2): p. 201-12.
20. Giustarini, D., et al., *Protein glutathionylation in erythrocytes.* Clin Chem, 2003. **49**(2): p. 327-30.
21. Niwa, T., et al., *Increased glutathionyl hemoglobin in diabetes mellitus and hyperlipidemia demonstrated by liquid chromatography/electrospray ionization-mass spectrometry.* Clin Chem, 2000. **46**(1): p. 82-8.
22. Muscat, J.E., et al., *Enhanced protein glutathiolation and oxidative stress in cigarette smokers.* Free Radic Biol Med, 2004. **36**(4): p. 464-70.
23. Sampathkumar, R., et al., *Increased glutathionylated hemoglobin (HbSSG) in type 2 diabetes subjects with microangiopathy.* Clin Biochem, 2005. **38**(10): p. 892-9.
24. Olivieri, O., et al., *Oxidative damage and erythrocyte membrane transport abnormalities in thalassemias.* Blood, 1994. **84**(1): p. 315-20.
25. Bateman, R.M., et al., *Erythrocyte deformability is a nitric oxide-mediated factor in decreased capillary density during sepsis.* Am J Physiol Heart Circ Physiol, 2001. **280**(6): p. H2848-56.
26. Cheng, M.L., et al., *Antioxidant deficit and enhanced susceptibility to oxidative damage in individuals with different forms of alpha-thalassaemia.* Br J Haematol, 2005. **128**(1): p. 119-27.
27. Rossi, R., et al., *Membrane skeletal protein S-glutathionylation and hemolysis in human red blood cells.* Blood Cells Mol Dis, 2006. **37**(3): p. 180-7.
28. Riederer, I.M. and B.M. Riederer, *Differential protein labeling with thiol-reactive infrared DY-680 and DY-780 maleimides and analysis by two-dimensional gel electrophoresis.* Proteomics, 2007. **7**(11): p. 1753-6.
29. Ringrose, J.H., et al., *Highly efficient depletion strategy for the two most abundant erythrocyte soluble proteins improves proteome coverage dramatically.* J Proteome Res, 2008. **7**(7): p. 3060-3.
30. Bradford, M.M., *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.* Anal Biochem, 1976. **72**: p. 248-54.
31. Dalle-Donne, I., et al., *Protein carbonyl groups as biomarkers of oxidative stress.* Clin Chim Acta, 2003. **329**(1-2): p. 23-38.
32. Dalle-Donne, I., et al., *Biomarkers of oxidative damage in human disease.* Clin Chem, 2006. **52**(4): p. 601-23.
33. Oliver, C.N., et al., *Age-related changes in oxidized proteins.* J Biol Chem, 1987. **262**(12): p. 5488-91.
34. Agarwal, S. and R.S. Sohal, *Relationship between susceptibility to protein oxidation, aging, and maximum life span potential of different species.* Exp Gerontol, 1996. **31**(3): p. 365-72.
35. Chao, C.C., Y.S. Ma, and E.R. Stadtman, *Modification of protein surface hydrophobicity and methionine oxidation by oxidative systems.* Proc Natl Acad Sci U S A, 1997. **94**(7): p. 2969-74.
36. Kriebardis, A.G., et al., *Membrane protein carbonylation in non-leukodepleted CPDA-preserved red blood cells.* Blood Cells Mol Dis, 2006. **36**(2): p. 279-82.
37. Kriebardis, A.G., et al., *Progressive oxidation of cytoskeletal proteins and accumulation of denatured hemoglobin in stored red cells.* J Cell Mol Med, 2007. **11**(1): p. 148-55.

38. Antonelou, M.H., et al., *Red blood cell aging markers during storage in citrate-phosphate-dextrose-saline-adenine-glucose-mannitol.* Transfusion, 2010. **50**(2): p. 376-89.
39. Barelli, S., et al., *Oxidation of proteins: Basic principles and perspectives for blood proteomics.* Proteomics Clin Appl, 2008. **2**(2): p. 142-57.
40. Madian, A.G. and F.E. Regnier, *Proteomic identification of carbonylated proteins and their oxidation sites.* J Proteome Res, 2010. **9**(8): p. 3766-80.
41. Levine, R.L., et al., *Determination of carbonyl content in oxidatively modified proteins.* Methods Enzymol, 1990. **186**: p. 464-78.
42. Yan, L.J., *Analysis of oxidative modification of proteins.* Curr Protoc Protein Sci, 2009. **Chapter 14**: p. Unit14 4.
43. Neuberg, C., A. Grauer, and B.V. Pisha, *The precipitation of carbonyl compounds with 2,4-dinitrophenylhydrazine - formation of isomeric dinitrophenylhydrazones.* Analytica Chimica Acta, 1952. **7**(3): p. 238-242.
44. Levine, R.L., et al., *Carbonyl assays for determination of oxidatively modified proteins.* Methods Enzymol, 1994. **233**: p. 346-57.
45. Reznick, A.Z. and L. Packer, *Oxidative damage to proteins: spectrophotometric method for carbonyl assay.* Methods Enzymol, 1994. **233**: p. 357-63.
46. Buss, H., et al., *Protein carbonyl measurement by a sensitive ELISA method.* Free Radic Biol Med, 1997. **23**(3): p. 361-6.
47. Smith, M.A., et al., *Cytochemical demonstration of oxidative damage in Alzheimer disease by immunochemical enhancement of the carbonyl reaction with 2,4-dinitrophenylhydrazine.* J Histochem Cytochem, 1998. **46**(6): p. 731-5.
48. Yan, L.J., W.C. Orr, and R.S. Sohal, *Identification of oxidized proteins based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunochemical detection, isoelectric focusing, and microsequencing.* Anal Biochem, 1998. **263**(1): p. 67-71.
49. D'Alessandro, A., et al., *Time-course investigation of SAGM-stored leukocyte-filtered red bood cell concentrates: from metabolism to proteomics.* Haematologica, 2012. **97**(1): p. 107-15.
50. van Gestel, R.A., et al., *Quantitative erythrocyte membrane proteome analysis with Blue-native/SDS PAGE.* J Proteomics, 2010. **73**(3): p. 456-65.
51. Bruce, L.J., et al., *A band 3-based macrocomplex of integral and peripheral proteins in the RBC membrane.* Blood, 2003. **101**(10): p. 4180-8.
52. Harboe, M., *A method for determination of hemoglobin in plasma by near-ultraviolet spectrophotometry.* Scand J Clin Lab Invest, 1959. **11**: p. 66-70.
53. Noe, D.A., V. Weedn, and W.R. Bell, *Direct spectrophotometry of serum hemoglobin: an Allen correction compared with a three-wavelength polychromatic analysis.* Clin Chem, 1984. **30**(5): p. 627-30.
54. Han, V., K. Serrano, and D.V. Devine, *A comparative study of common techniques used to measure haemolysis in stored red cell concentrates.* Vox Sang, 2010. **98**(2): p. 116-23.
55. Burton, N.M. and L.J. Bruce, *Modelling the structure of the red cell membrane.* Biochem Cell Biol, 2011. **89**(2): p. 200-15.
56. Koch, C.G., et al., *Duration of red-cell storage and complications after cardiac surgery.* N Engl J Med, 2008. **358**(12): p. 1229-39.
57. Neelam, S., et al., *Functional 20S proteasomes in mature human red blood cells.* Exp Biol Med (Maywood), 2011. **236**(5): p. 580-91.

58. Fagan, J.M. and L. Waxman, *The ATP-independent pathway in red blood cells that degrades oxidant-damaged hemoglobin.* J Biol Chem, 1992. **267**(32): p. 23015-22.
59. Rubin, O., et al., *Microparticles in stored red blood cells: an approach using flow cytometry and proteomic tools.* Vox Sang, 2008. **95**(4): p. 288-97.
60. Bosman, G.J., et al., *The proteome of red cell membranes and vesicles during storage in blood bank conditions.* Transfusion, 2008. **48**(5): p. 827-35.
61. Kriebardis, A.G., et al., *RBC-derived vesicles during storage: ultrastructure, protein composition, oxidation, and signaling components.* Transfusion, 2008. **48**(9): p. 1943-53.

PART 3.

CHARACTERIZATION OF THE STORAGE-INDUCED OXIDIZED PROTEOMES OF RED BLOOD CELLS

Table of contents

List of illustrations

List of tables

Introduction

RBCs were clearly shown to be challenged by oxidative modification *in vivo* as well as during their *in vitro* storage as ECs (see general introduction and part 2). Detection of carbonylation and cysteine oxidation allowed proving the occurrence of protein oxidative modifications inside RBCs, as storage duration- and cellular compartment-dependent phenomena. Proteomic studies conducted so far in the transfusion medicine field mainly focused on global storage-induced changes in erythrocyte proteome. Of particular interest is to question the identity of proteins or protein function families that are particularly affected by above-mentioned modifications. One can legitimately wonder whether all proteins in a given cellular compartment are equally susceptible to oxidation, or if oxidative alterations target specific proteins, thus altering some target molecular functions inside the cell. EC storage-related oxidized proteins compose a sub-proteome that needs to be characterized in time-dependent and localization-dependent manners, in order to bring new clues enabling a better understanding of biochemical and rheological changes occurring during the RBCs storage, thus potentially allowing the development of new storage conditions for a better efficiency of transfused blood products.

Erythrocytes are considered as a model of choice for multiple studies due to their relatively low complexity. Indeed, their maturation process includes the depletion of all intracellular organelles, making it easy to investigate cell membranes in particular. However, RBCs extracts are complex samples regarding proteomic tools for protein identification (*i.e.* mass spectrometry, MS). In particular, the dynamic range of protein concentrations inside a sample is critical for a good proteome analysis. To investigate sub-proteomes from such complex samples, it is necessary to fractionate samples before analysis. In Part 2, the interest of subcellular fractionation and electrophoresis protein separation for a better visualization of proteins of interest was shown. As for MS-based protein identification, the rule is the same: the lower the sample complexity, the better the identification. Sub-proteome profiling can rely on three approaches: physico-chemical isolation (2DE for instance), cellular fractionation (as the subcellular fractionation developed in the part 2 of this PhD thesis), or chemical targeting by specific probes (*e.g.* enrichment of a particular PTM-related sub-proteome).

A sub-proteome-targeted approach is based on MS-coupled affinity purification (AP-MS). The AP step can be conducted through antibody/antigen affinity (immunopurification) or other ligands such as oligonucleotides, proteins or chemicals. Carbonylated proteins enrichment has been realized with derivatives of hydrazines, Girard's P reagent or O-ECAT labeling. The group of Fred E. Regnier successfully investigated carbonylated proteins from bovine serumalbumine (BSA) solution [1], yeast extract [2, 3] or rat plasma [4] through biotin-hydrazide derivatization followed by avidin-affinity based fluorescent labeling and MALDI-MS/MS identification [3] or chromatographic purification and ESI-MS/MS identification [1, 2, 4]. This group also made use of hydrazide derivative Girard's P reagent (GPR, 1-(2-hydrazino-2-oxoethyl)pyridinium chloride) enabling the purification of derivatized peptides by strong cation exchange chromatography (SCX) [5]. GPR derivatization provides a quaternary amine function allowing ionic interaction between the positively charged derivatized peptides and a negatively charged solid phase. Widely used for spectrophotometric quantitation of carbonylation content, 2,4-DNPH derivatization was also used as first step of carbonylome immunoprecipitation to study apoptosis induction in the human promyelocytic leukemia HL60 cell line [6]. Also, ICAT-like reagent O-ECAT standing for oxidation-dependent element coded affinity tags was developed by Meares and coworkers to assess carbonylome through AP coupled to nano flow liquid chromatography and fourier transform ion cyclotron resonance-MS (AP-nanoLC-FT-ICR-MS) analysis on recombinant human serum albumin [7]. For a methodological review on ways of investigating the carbonylated proteins, see reference [8].

Several groups have thus studied the carbonylated proteome of some prokaryote or eukaryote organisms, as a response to *in vitro*-induced oxidative stress as well as an *in vivo* pathological or aging biomarker [9-11]. Investigating protein carbonylation in the case of RBCs storage is a little bit particular in the sense that it is both an unprovoked and non-physiological modification. As mentioned in Part 2, this PTM is widely investigated as a quantitative biomarker of oxidative stress and aging, including in the transfusion-purposed RBC storage. However, the following work is the first time that a time-dependent characterization of this sub-proteome is assessed in this field. To this purpose, two additional separation steps were included over the subcellular protein segregation. Modified proteins were isolated by affinity purification before 1D-PAGE,

and digested peptides were separated based on hydrophobicity prior to MS/MS analysis.

As for proteins prone to cysteine oxidation, the different approaches described in Part 2 allowed some groups to investigate redox-sensitive proteomes from diverse cellular origin, either as a response of *in vitro* stimulation or as pathology biomarker. Mainly proteins involved in metabolism, redox homeostasis, signal transduction, cytoskeleton or vesicle trafficking appeared sensitive to reversible cysteine sulfenation [12-14]. In particular, these protein functions were shown targeted in a mice model presenting a peroxiredoxin II knock out [15]. Spots of interest detected in Part 2 will be here analyzed for protein identification and bioinformatics-based determination of targeted function families.

Chapter one

Characterization of the red blood cell storage-dependent thiol-reactive proteome

This chapter refers to the article

"Cysteine redox proteomics of the hemoglobin-depleted cytosolic fraction of stored red blood cells"

In preparation

Study design

This study directly follows the investigation on the evolution of the oxidation of protein cysteine residues (see Part 2, Chapter 1). Briefly, five ECs were followed throughout the storage period, and reversibly oxidized cysteine residues were labeled by IR maleimide dyes. Redox-DIGE analyses allowed discriminating several protein populations according to the evolution of their cysteine-related oxidative state. Spots of interest were excised from Coomassie Blue stained 2D-gels and contained proteins were digested prior to peptide fractionation and sequencing through LC-MS/MS experiments. Proteins corresponding to differentially storage-affected cysteine residues were identified by database searching. Proteomes corresponding to the different categories of oxidation evolution were analyzed relatively to engaged molecular functions. The workflow is pictured out on Figure 1 in Part 2, Chapter 1.

Material and methods

Spot excision, *in-gel* tryptic digestion and peptide extraction

Spots of interest were manually excised as depicted in Figure 23, from Coomassie Blue-stained 2D gel corresponding to the mix [Day 6 + Day 41] from the EC5 (see details in annex A). The gel pieces were then washed in 50/50 ethanol/50 mM ammonium bicarbonate (AB) pH 8.4, until getting destained. Proteins were then reduced and alkylated during one hour with 10 mM dithioerythritol (DTE) in 50 mM AB pH 8.4 and during 45 minutes with 55 mM iodoacetamide (IAA) in 50 mM AB pH 8.4, respectively, at 37 °C under shaking and protected from light. Proteins were digested by incubating the spots in a 12.5 ng/µL trypsin solution in 50 mM AB pH 8.4, 10 mM calcium chloride, over night at 37 °C under shaking. Tryptic peptides were finally extracted from the spots by shrinking the gel pieces in a 5 % formic acid (FA) solution containing increasing concentrations of ethanol. Peptide extracts were vacuum centrifuged upon dryness, washed in 100 % acetonitrile (ACN), and stored as dried pellets at -30 °C until use.

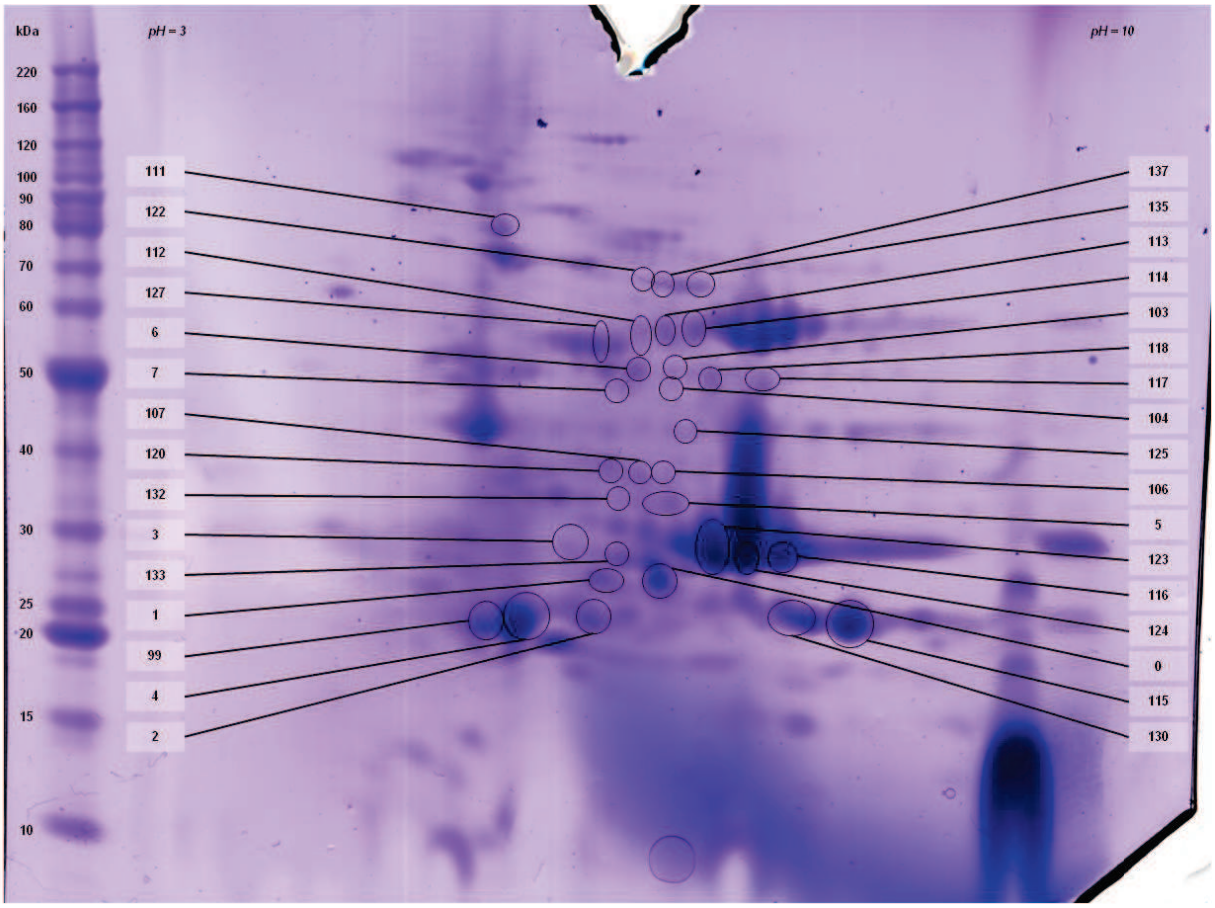


Figure 23: 2DE spot excision for MS-based identification of proteins presenting storage-related changes in cysteine oxidation status. Spots of interest were detected thanks to densitometric analysis of 2D gels from 5 biological replicates (10 2D gels, giving access to 20 2D gel images, see details in Part 2 Chapter 1), and were excised from coomassie-blue stained 2D gel of the mix [day 6 + day 41] from EC5. First dimension was a 7 cm long 3-10 non linear pH gradient and 2nd dimension was a 4-15 % polyacrylamide precast minigel. Spot tags are the matchID assigned by ImageMaster during the manual spot matching step. Labels on the left side of the gel are molecular weights (in kDa) of the protein ladder.

LC-MS/MS analysis

Proteins identification was carried out on a LC system (Dionex™ UltiMate™ 3000-RSLCnano System, Thermo Scientific, Rockford, IL, USA) coupled to a MS (amaZon ETD, Bruker Daltonics, Bremen, Germany). Peptide extracts were resuspended in 2 % ACN, 20 % FA, and halves of extracts were loaded on a Dionex Acclaim PepMap RSLC column (300 µm x 15 cm, C18, 2 µm, 100 Å) at a 4 µL/min rate in 95 % eluent A (0.1 % FA aqueous solution) and 5 % of eluent B (dH_2O/CAN/FA 2/8/0.8). Peptides were separated through a 40 minutes gradient, rising to 25 % of eluent B in 30 minutes, then to 40 % in 5 minutes, and finally to 90 % in 10 minutes. Mass spectra of separated peptides were acquired in the positive scan mode from 300 to 1500 m/z.

An automatic MS/MS CID fragmentation was performed on the three most intense precursor ions of each spectrum. Precursor ions were excluded from MS/MS fragmentation after appearance in 1 spectrum, and released after 0.15 minute. Precursors presenting a 5 % increase in instensity during their exclusion time were reconsidered for fragmentation then again excluded.

Data processing, database query and functional comparison

Once an LC-MS/MS run has finished, data are automatically processed in DataAnalysis (Bruker software for LC-MS data analysis). The process consists in compounds detection, mass spectra deconvolution, and exportation of the compound list. Compounds are all detected peaks. Parameters for automatic data processing were set as follows: only the 2000 most intense compounds were taken into account.

Spectra were submitted *via* Mascot to database search with the following parameters: Database = SwissProt; Taxonomy = Homo sapiens (human); Enzyme = Trypsin, allow up to 1 missed cleavage; Variable modifications = Methionine oxidation, Cysteine NEM-alkylation, Cysteine DY680 maleimide labeling, Cysteine DY780 maleimide labeling; Peptide tolerance = ± 0.35 Da; MS/MS tolerance = ± 0.35 Da; Peptide charge = 2+, 3+ and 4+. Proteins were automatically validated for Mascot scores above 40, and a manual validation was applied to consider only proteins identified from at least 2 peptides.

Identified proteins were submitted to GO analysis using Blast2GO®, an on-line software for functional annotation of sequences and analysis of annotation data (www.blast2go.com) [16]. Protein fasta sequences were thus loaded, submitted to blast search, mapped and annotated (GO annotation, InterPro annotation and enzyme code annotation) in order to assign molecular functions to sequences. Sub-proteomes corresponding to the previously determined categories of evolution of cysteine residues redox state (see Part 2 Chapter 1) were compared based on molecular function.

Results and discussion

Among the 32 spots presenting storage-related variation in IR signal (labeling of reversibly oxidized cysteines), 43 proteins were identified. Table 2 summarizes the identification of these proteins, presenting the percentage of sequence coverage, the number of sequenced peptides, and the Mascot score attributed to the identification. For each protein, these parameters are shown for its global identification (merged LC-MS/MS data from all spots, proteins classified by accession number) as well as in an individual spot manner (for each protein, the spots in which it has been identified are organized based on the highest Mascot score).

It appears that some proteins were identified in several spots that presented different evolution of labeling intensity over the storage period. This can be due to the proximity of spots on mini 2D gels, making possible the detection of traces of a protein in adjacent spots. Also, the existence of protein isoforms (variants of a protein presenting p*I* shifts on 2D gels) allows the detection of a protein in several distinct spots. Finally, even if the p*I*-mass combination gives a good protein separation, spots can easily contain several proteins, sharing identical p*I*-mass couple, or at least showing the same migration behavior in the two dimensions. Two or more proteins being differentially affected by cysteine oxidation along the RBC storage can thus be found in a single spot. The most abundant protein can thus mask the labeling signal of others, and all proteins in such a spot can be falsely assigned to a given category of evolution of cysteine oxidation. For instance, the protein flavin reductase (NADPH) was identified in spots belonging to each of the four categories of evolution of cysteine oxidation (see Table 2). However, it was much better identified in the “up” category, regarding number of sequenced peptides and sequence coverage, though these parameters cannot be considered as quantitative.

In Part 2 Chapter 1, the spots of interest were classified into 4 categories corresponding to different evolution of signal intensity, thus different evolution of cysteine oxidation status throughout the RBCs storage period. As shown in Figure 24, more than half (56 %) of identified proteins present a category-related specificity. Particularly, 30 % and 16 % of identified proteins

are specific to the “down” and “up” categories, respectively. Moreover, it is noteworthy that only one protein, the above-mentioned flavin reductase (NADPH), was commonly identified in all the four determined categories. These elements are interestingly concordant with our working hypothesis about protein sub-populations being differentially affected by oxidative damages during RBC storage.

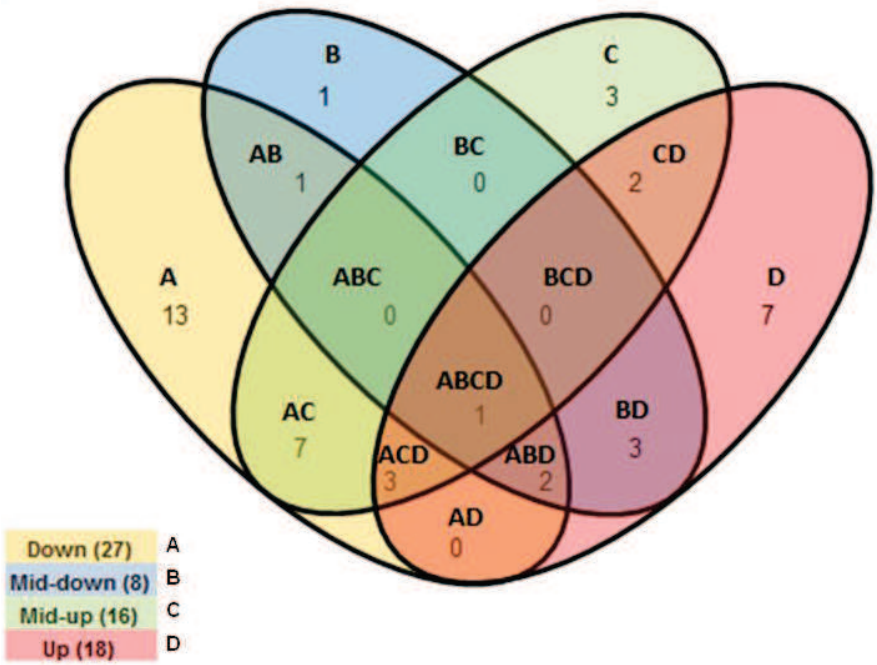


Figure 24: Venn diagram of identified proteins showing a storage-induced change in their cysteine oxidation state. 43 proteins were identified, of which 55 % are category-specific.

The molecular functions involved through identified proteins were investigated by gene ontology annotation (GO annotation), and are presented in Figure 25. From the list of all the 43 identified proteins presenting variation of cysteine redox status over storage, it appears that binding and catalytic activities were the two mostly represented molecular functions, corresponding to 36.7 and 30.3 % of assigned functions, respectively (see Figure 25A). Antioxidant and enzyme regulation activities were also well represented with both 6.4 % of assigned functions (see Figure 25A). When looking at proteins identified in each category, some

changes in molecular function proportions were clearly observed. Proteins belonging to the "down" category (decrease of labeling signal over storage, see Figure 25B) showed an increase in enzyme regulation activity (+32 %). However, dealing with proteins specific to this category, it appears that antioxidant and enzyme regulation activities were down-represented (-42% each), and catalytic activity up-represented (+35 %), as shown in Figure 25F. Concerning the "mid-down" category (decrease of labeling signal until day 27 then increase until day 41), the whole pool of identified proteins showed an increase in proportions of catalytic (+65 %) and antioxidant (+95 %) activities (see Figure 25C). Only one protein was specifically identified in this category (L-lactate dehydrogenase B chain) and the annotation process revealed equally distributed binding, catalytic and structural molecule activities (see Figure 25G). Dealing with the "mid-up" category (increase of labeling signal until day 27 then decrease until day 41), the proportions of the four above-mentioned mostly represented molecular functions are increased by 17 %, 18 %, 11 % and 67 %, respectively. Others under-represented molecular functions were not assigned in this category, except transporter activity (3.57 %, see Figure 25D). Only three proteins were specific to the "mid-up" category (coiled-coil domain-containing protein 158, probable phosphoglycerate mutase 4 and selenium-binding protein 1). These 3 proteins, as shown in Figure 25H, only involved binding and catalytic activities, increased by 82 % and 10 %, respectively, in comparison to the mean global molecular functions repartition. Finally, the "up" category (increase of the labeling signal from day 6 to day 41) showed nearly the same mean distribution of molecular functions, except for the antioxidant activity which was increased by 91% and the enzyme regulation activity which was lowered by 36 % (see Figure 25E). As for the 7 proteins specifically identified in this category, Figure 25I shows that the catalytic and enzyme regulation activities were decreased by 21 and 26% respectively, and that the antioxidant and transporter activities were increased by 48 and 107 %, respectively.

To assess molecular functions differentially affected by cysteine oxidation, it is better to work with data that are specific to the categories of evolution of this oxidative modification. Due to the low number of proteins specifically identified in the "mid-down" and "mid-up" categories, the proportions of engaged molecular functions in these two groups will not be taken into account. As for the "down" group, where IR labeling signal of reversibly oxidized cysteines

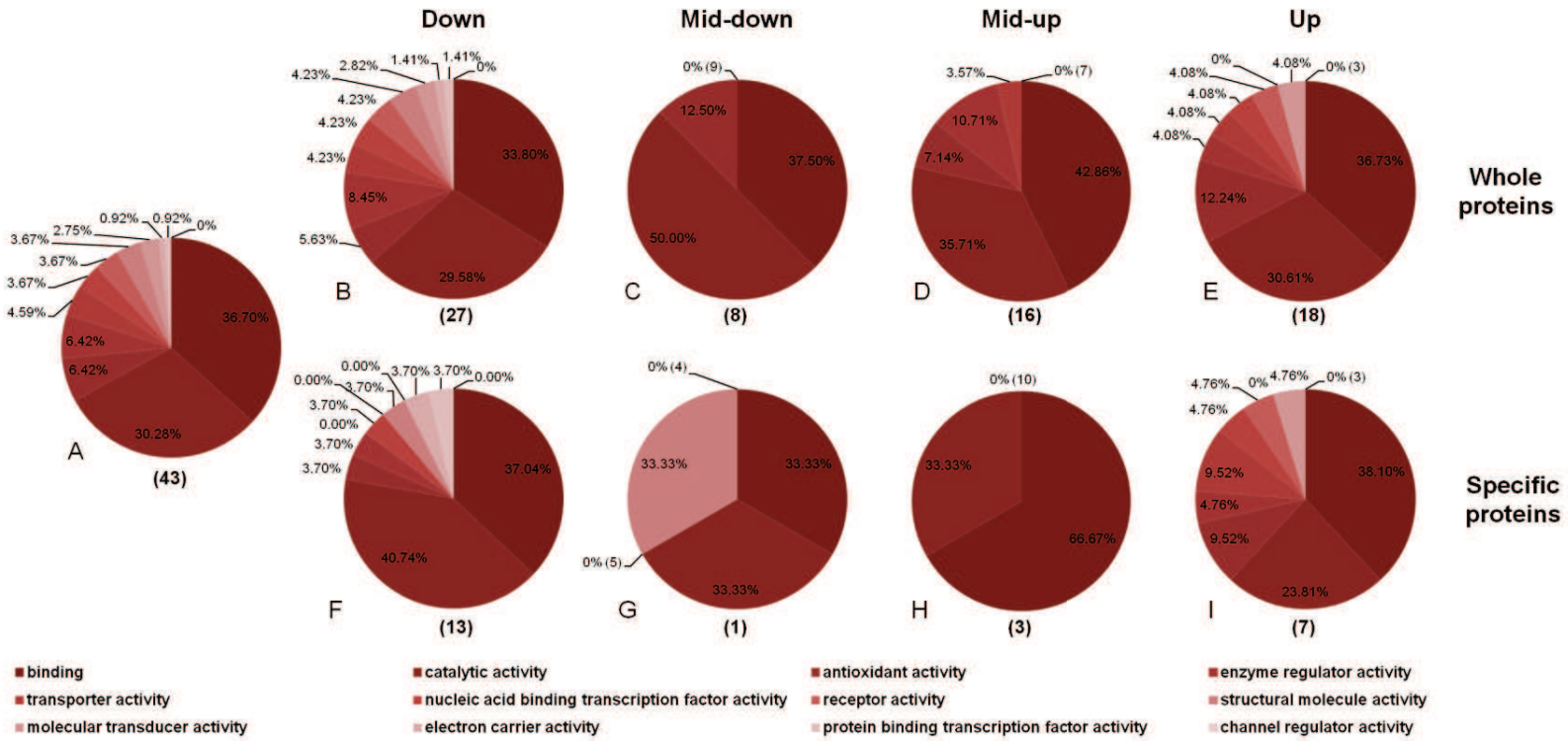


Figure 25: Repartition of molecular functions in differentially cysteine-oxidized protein populations in stored RBCs. Diagram A represent the repartition in the whole identified cysteine redox proteome. Diagrams B to I represent the repartitions in the "down" (B and F), "mid-down" (C and G), "mid-up" (D and H) and "up" (E and I) categories, either for all proteins identified in these categories (B to E) or for proteins specific to each category (F to I). The bracketed numbers following some "0 %"-labels indicate that number of following unrepresented molecular functions, according to the ranking in the legend. Under each diagram is the number of identified proteins. The color shading in the legend should be read from left to right then top to down.

tended to decrease or disappear with the storage period, the hypotheses are either the increase of irreversible sulfonic cysteine oxidation state and/or the degradation of these oxidized proteins. Catalase was the unique protein involved in RBC antioxidant defenses to be specifically identified in this group. It represented a lower part of antioxidant (AO) proteins than in the entire group of identified proteins (9 % of annotated sequences *vs.* 17 %). Moreover, despite the higher abundance of proteins showing a catalytic activity (100 % of annotated sequences *vs.* 82 %), proteins related to a catalytic oxidoreductase (OR) activity were lowered in this group (27 % of annotated sequences *vs.* 32 %). Some oxidoreductases are involved in direct as well as indirect AO defenses. Here, three OR proteins were identified: catalase, biliverdin reductase A (BIEA) and GDP-L-fucose synthase (FCL). The former is part of RBCs AO defenses, dismutating hydrogen peroxide into water and molecular oxygen [17]. The second is involved in porphyrin metabolism, allowing the NAD(P)H-dependent reduction of the hemoglobin-catabolism product biliverdin into bilirubin [18]. The latter acts in the fructose and mannose metabolism pathway, converting GDP-4-oxo-6-deoxy-D-mannose into GDP-L-fucose, in an NADPH-dependent manner [19]. MS/MS data that allowed us to identify the catalase protein revealed sulfonic acid state on Cys-460 from day 41 samples, but not from days 6 and 27 ones, from which only chemical alkylation of Cys-460 was observed (data not shown). This means that catalase Cys-460 was either unmodified or reversibly oxidized during the 4 first weeks of storage, then became irreversibly oxidized by the end of RBCs banking, supporting the redox-DIGE information of a storage time-dependent irreversible cysteine oxidation. As for BIEA and FCL, MS/MS analysis did not reveal cysteine sulfonic acid state due to the absence of sequenced cysteine-containing peptide. These two proteins were also identified in other analyses that did not involve any chemical cysteine modification. No Cys-peptide was sequenced as well. It is possible that intrinsic properties of these Cys-peptides prevent their correct ionization thus detection by MS. Another explanation could be that other storage-related modifications, such as the carbonylation of amino acids in the near area of cysteines, prevent the good MS/MS assignment of these peptides.

Contrary to the “down” category, proteins from the “up” group, characterized by an increase of the IR labeling signal, could find an explanation in the accumulation of reversibly cysteine-

oxidized protein copies. This group was characterized by a decrease in catalytic activity proportion (62 % of annotated sequences *versus* 82 % for the pool of all the 43 identified proteins in this study) and an increase in AO activity proportion (25 % of annotated sequences *versus* 17 %). As for the catalytic activities, of interest is the absence of constituent of isomerase and transferase activities in comparison to the mean function distribution (were 12 % and 20 % of annotated sequences, respectively). However, the catalytic lyase activity proportion was found increased (25 % of annotated sequences *versus* 15 %). Peroxiredoxin-1 (Prdx1) and protein DJ-1 were the two identified constituents of both OR and peroxidase antioxidant activity groups. If the proportion of the former was found to be slightly decreased in the “up” category, the latter was found increased by a factor 2 (both 25 % of annotated sequences *versus* 32 and 12 %, respectively). Prdx1 is a 2-cysteines thioredoxin peroxidase that reduces peroxides with concomitant homodimerization through sulfenyl formation at the peroxidatic cysteine (Cys-52) and inter-molecular disulfide bridging with the resolving cysteine (Cys-173) of another subunit [20]. The enzyme is regenerated *via* reduction by thioredoxin. In the case of Prdx1, cysteines are thus essential for the antioxidant activity. It can happen that the peroxidatic cysteine (Cys-52) goes further oxidized into sulfinic and even sulfonic states upon oxidative stress. The Cys-SO_2H can be reduced by sulfiredoxins whereas Cys-SO_3H cannot. Throughout the storage period, it seems that this enzyme was accumulating in still reversible oxidized form. It cannot be here distinguished whether the accumulation concerns Cys-SOH or Cys-SO_2H, so it can be hypothesized a progressive defect of thioredoxin and/or sulfiredoxin, preventing the regeneration of prdx1 in its active form.

As for the protein DJ-1, it is known to prevent oxidative stress-induced cell death by eliminating hydrogen peroxide in an atypical peroxiredoxin-like peroxidase activity [21] and by displaying a cysteine protease activity through removal of a 15-amino acid C-terminal peptide [22]. Protein DJ-1 contains three cysteine residues. In particular, the Cys-106 residue is responsible for cytoprotection against oxidative stress, and is easily oxidized (for a review on the role of cysteine oxidation in DJ-1 function, see [23]). Though it is not clearly stated in the literature, it can be hypothesized that Cys-106 oxidation would alter protein DJ-1 ability to take over H_2O_2 thus to protect cell from oxidative stress. Moreover, MS/MS analysis allowed detecting

carbamidomethylation of Cys-46 throughout the storage period (data not shown). This modification is the result of the chemical alkylation of reduced cysteines, and sustains the redox-DIGE information of reversible cysteine oxidation on this protein.

This proteomic analysis of storage-induced cysteine-oxidized soluble proteome revealed differences in the proportions of molecular functions engaged through reversibly or irreversibly oxidized proteins, as suggested by the different evolutions of labeling intensity shown in Part 2 Chapter 1. In particular, proteins presenting AO activity appear more prone to reversible oxidation of their cysteine-containing active sites, which would suggests a preserved AO activity potential in stored RBCs.

Table 2: Proteomic identification of proteins showing a storage-dependent variation of cysteine oxidation status.

Name	AC #	Global analysis			Spots analysis				
		Seq. cov. (%)	Peptide #	ID score	Match ID	Intensity evolution	Seq. cov. (%)	Peptide #	ID score
Acylamino-acid-releasing enzyme	**ACPH_HUMAN**	**19.3**	**10**	**559.91**	**111**	**Down**	**16.5**	**10**	**558.91**
Actin, cytoplasmic 1	**ACTB_HUMAN**	**19.5**	**3**	**198.93**	**111**	**Down**	**9.9**	**3**	**179.83**
Retinal dehydrogenase 1	**AL1A1_HUMAN**	**38.1**	**13**	**497.33**	113	Mid-up	25.1	11	457.03
					114	Down	17.2	6	291.99
					112	Down	9.8	4	192.90
Serum albumin	**ALBU_HUMAN**	**18.7**	**10**	**336.55**	127	Mid-up	12.6	8	264.99
					137	Down	4.3	2	114.09
					113	Mid-up	3.1	2	112.19
					5	Up	4.8	3	111.33
					133	Up	1.8	1	98.67
					120	Down	1.8	1	69.13
					4	Down	3.0	2	68.40
					114	Down	3.1	2	61.35
					125	Down	1.8	1	56.97
					104	Down	1.8	1	56.93
Apolipoprotein A-I	**APOA1_HUMAN**	**20.2**	**4**	**157.07**	**106**	**Down**	**13.5**	**3**	**100.29**
					120	Down	8.6	2	90.16
					117	Down	4.5	1	49.23
Biliverdin reductase A	**BIEA_HUMAN**	**25.3**	**5**	**183.95**	**107**	**Down**	**16.6**	**4**	**161.61**
Flavin reductase (NADPH)	**BLVRB_HUMAN**	**65.0**	**12**	**857.49**	115	Up	65.0	12	835.29
					130-2	Up	50.5	11	738.02
					130-1	Up	50.5	9	542.79
					0	Mid-up	18.9	4	168.42
					103	Down	21.8	2	133.90
					132	Mid-down	7.3	1	59.40

Name	AC #	Global analysis			Spots analysis				
		Seq. cov. (%)	Peptide #	ID score	Match ID	Intensity evolution	Seq. cov. (%)	Peptide #	ID score
Carbonic anhydrase 1	**CAH1_HUMAN**	**70.9**	**15**	**1002.17**	**124**	**Mid-up**	**61.7**	**14**	926.13
					116	Up	70.9	14	869.77
					123	Mid-up	70.9	14	794.63
					5	Up	24.1	6	312.43
					130-1	Up	13.4	4	223.78
					118	**Down**	**15.3**	**5**	**221.60**
					117	Down	8.0	3	163.65
					133	Up	8.8	2	118.31
					115	Up	11.9	3	117.01
					0	Mid-up	8.0	2	99.41
					114	Down	4.6	1	49.54
Carbonic anhydrase 2	**CAH2_HUMAN**	**7.3**	**2**	**106.03**	**116**	**Up**	**7.3**	**2**	**106.03**
Carbonic anhydrase 3	**CAH3_HUMAN**	**21.2**	**2**	**107.35**	**116**	**Up**	**11.2**	**2**	**107.35**
Catalase	**CATA_HUMAN**	**26.9**	**10**	**462.88**	**114**	**Down**	**15.7**	**7**	**358.56**
					118	Down	**12.5**	**6**	**247.83**
					117	Down	1.9	1	42.75
Coiled-coil domain-containing protein 158	**CD158_HUMAN**	**1.4**	**1**	**70.18**	**123**	**Mid-up**	**0.8**	**1**	**41.51**
Alpha-enolase	**ENOA_HUMAN**	**30.9**	**11**	**445.35**	**117**	**Down**	**29.5**	**10**	**435.09**
					118	Down	18.9	6	199.36
GDP-L-fucose synthase	**FCL_HUMAN**	**16.2**	**4**	**124.85**	**107**	**Down**	**11.8**	**3**	**113.30**
Fibrinogen beta chain	**FIBB_HUMAN**	**31.6**	**8**	**495.35**	114	Down	17.7	7	452.84
					113	Mid-up	10.4	5	167.51
Fumarate hydratase, mitochondrial	**FUMH_HUMAN**	**2.4**	**1**	**42.43**	**117**	**Down**	**2.4**	**1**	**42.43**
Glucose-6-phosphate 1-dehydrogenase	**G6PD_HUMAN**	**4.7**	**1**	**99.92**	**113**	**Mid-up**	**2.1**	**1**	**66.42**
					114	Down	2.1	1	50.42
Rab GDP dissociation inhibitor beta	**GDIB_HUMAN**	**50.6**	**17**	**778.93**	6	Mid-up	44.5	17	755.19
					103	Down	17.1	5	192.42
					104	Down	2.5	1	55.87
Hydroxyacylglutathione hydrolase, mitochondrial	**GLO2_HUMAN**	**6.5**	**2**	**73.96**	**116**	**Up**	**6.5**	**2**	**73.96**
Glutathione S-transferase omega-1	**GSTO1_HUMAN**	**33.4**	**8**	**327.32**	**133**	**Up**	**34.4**	**8**	**259.97**
					3	Mid-down	25.7	6	229.39
Delta-aminolevulinic acid dehydratase	**HEM2_HUMAN**	**8.8**	**2**	**108.84**	132	Mid-down	**8.8**	**2**	108.84
					5	Up	8.8	2	78.77
					107	Down	8.8	2	67.98
Hypoxanthine-guanine phosphoribosyltransferase	**HPRT_HUMAN**	**33.5**	**6**	**368.47**	**1**	**Down**	**26.1**	**6**	**317.24**
					0	Mid-up	20.2	4	226.25
Heat shock cognate 71 kDa protein	**HSP7C_HUMAN**	**5.0**	**2**	**92.12**	**111**	**Down**	**3.4**	**2**	**75.70**

Name	AC #	Global analysis			Spots analysis				
		Seq. cov. (%)	Peptide #	ID score	Match ID	Intensity evolution	Seq. cov. (%)	Peptide #	ID score
L-lactate dehydrogenase B chain	**LDHB_HUMAN**	**19.2**	**1**	**156.76**	**132**	**Mid-down**	**4.5**	**1**	**140.79**
Malate dehydrogenase, cytoplasmic	**MDHC_HUMAN**	**16.2**	**5**	**187.09**	**5** 132	**Up** Mid-down	**13.2** 9.6	**4** 3	**148.92** 81.44
Protein DJ-1	**PARK7_HUMAN**	**43.4**	**5**	**172.01**	**2**	**Up**	**35.4**	**5**	**172.01**
Phosphatidylethanolamine-binding protein 1	**PEBP1_HUMAN**	**35.3**	**4**	**137.80**	**115**	**Up**	**27.3**	**4**	**137.80**
Probable phosphoglycerate mutase 4	**PGAM4_HUMAN**	**12.6**	**3**	**153.89**	**124** 123	**Mid-up** Mid-up	**12.6** 12.6	**3** **2**	**133.88** **76.55**
Protein-L-isoaspartate(D-aspartate) O-methyltransferase	**PIMT_HUMAN**	**21.6**	**4**	**162.99**	**1**	**Down**	**21.6**	**4**	**162.99**
Phosphatidylinositol transfer protein alpha isoform	**PIPNA_HUMAN**	**5.9**	**1**	**53.38**	**5**	**Up**	**3.7**	**1**	**53.38**
Bisphosphoglycerate mutase	**PMGE_HUMAN**	**37.5**	**7**	**397.80**	**133** 0	**Up** Mid-up	**29.7** 7.3	**7** 1	**397.80** 40.84
Purine nucleoside phosphorylase	**PNPH_HUMAN**	**21.5**	**6**	**244.70**	**132** 5	**Mid-down** Up	**21.5** 11.8	**6** 3	**224.03** 95.24
Peroxiredoxin-1	**PRDX1_HUMAN**	**16.1**	**3**	**153.44**	**115**	**Up**	**13.6**	**3**	**111.92**
Peroxiredoxin-2	**PRDX2_HUMAN**	**49.0**	**10**	**683.10**	**4** 2 99	**Down** Up Mid-down	**40.9** 40.9 40.9	**10** 10 10	**614.71** 561.12 549.03
Peroxiredoxin-6	**PRDX6_HUMAN**	**77.7**	**20**	**1164.10**	**0** **1** 133	**Mid-up** **Down** Up	**74.1** **62.9** 15.6	**20** **14** 3	**1125.75** **688.44** 111.04
Serine/threonine-protein phosphatase 2A activator	**PTPA_HUMAN**	**20.9**	**4**	**169.31**	**120**	**Down**	**12.8**	**4**	**169.31**
Bifunctional purine biosynthesis protein PURH	**PUR9_HUMAN**	**15.2**	**5**	**202.17**	**135**	**Down**	**9.3**	**5**	**192.69**
Ras-related protein Rap-1b	**RAP1B_HUMAN**	**26.1**	**3**	**151.24**	99 4	Mid-down Down	17.9 6.5	3 1	151.24 96.61
Adenosylhomocysteinase	**SAHH_HUMAN**	**17.6**	**5**	**223.43**	**7** 6	**Down** Mid-up	**14.1** 6.7	**5** 3	**192.07** 97.80
Selenium-binding protein 1	**SBP1_HUMAN**	**44.9**	**15**	**743.20**	**127** 113	**Mid-up** Mid-up	**38.6** 11.9	**15** 5	**743.20** 230.92
Stress-induced-phosphoprotein 1	**STIP1_HUMAN**	**6.4**	**3**	**127.22**	**122** 137	**Down** Down	**5.2** 1.8	**3** 1	**87.08** 76.74
Transaldolase	**TALDO_HUMAN**	**9.8**	**2**	**105.41**	107 120	**Down** Down	3.3 6.5	1 2	68.50 60.24

Name	AC #	Global analysis			Spots analysis				
		Seq. cov. (%)	Peptide #	ID score	Match ID	Intensity evolution	Seq. cov. (%)	Peptide #	ID score
Triosephosphate isomerase	**TPIS_HUMAN**	**46.5**	**11**	**535.92**	**124**	**Mid-up**	**46.5**	**11**	**521.88**
					123	Mid-up	31.8	7	384.60
					133	Up	15.4	3	89.19
					0	Mid-up	5.2	1	59.62

Chapter two

Characterization and qualitative evolution of the storage-induced red blood cell carbonylome

This chapter refers to the article

"Proteomics of the red blood cells carbonylome during banking of erythrocyte concentrates"

In preparation

Study design

Soluble and membrane extracts from day 6, day 27 and day 41 collected ECs (see Annex A for details) were submitted to carbonyl derivatization with a biotin derivative, allowing their purification by mean of avidin-biotin affinity. Purified proteins were then separated by SDS-PAGE, and identified by LC-MS/MS analyses. Carbonylome was analyzed and compared at each storage duration, relatively to engaged molecular functions and biological processes. The workflow is described in Figure 26.

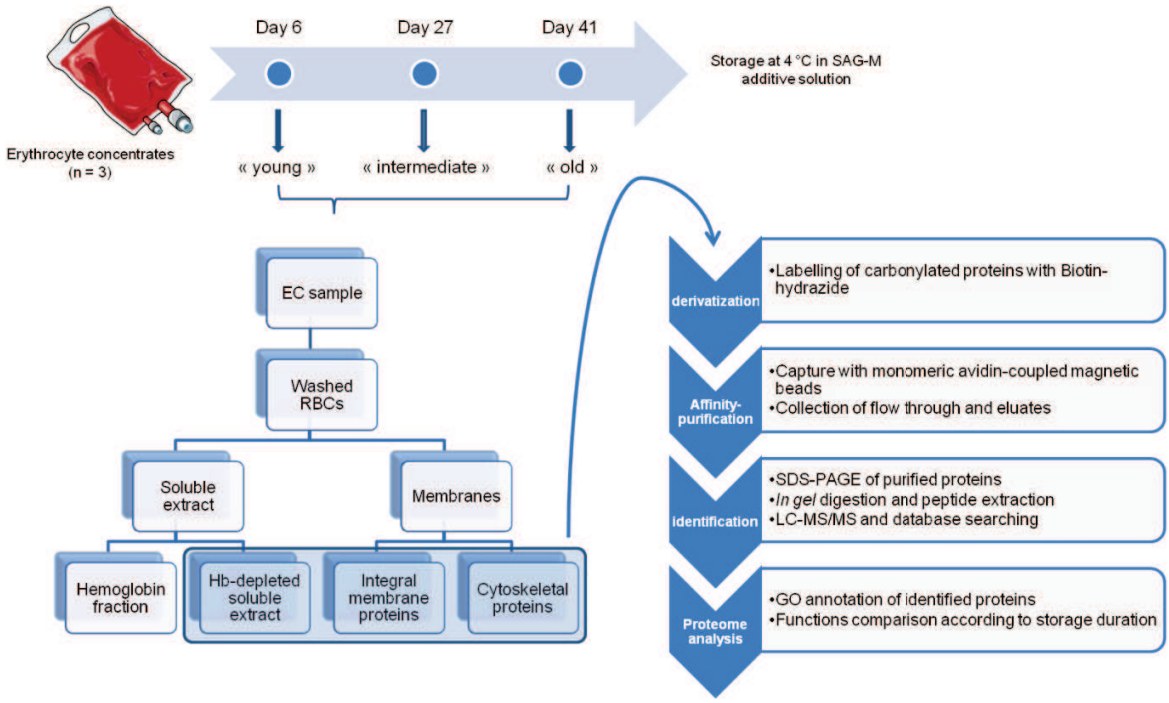


Figure 26: Study design for the analysis of the carbonylome in stored RBCs. Hb-depleted soluble fraction, integral protein membrane fraction and cytoskeleton membrane fraction were obtained from three ECs at days 6, 27 and 41 of the storage period. Proteins bearing functional carbonyl group(s) were derivatized with biotin-hydrazide and purified *via* affinity with monomeric avidin-coupled magnetic beads. Captured proteins were separated by SDS-PAGE and identified by LC-MS/MS analysis. Automatic GO annotation allowed the comparison stored RBCs carbonylome along the storage period.

Material and methods

Sample collection and preparation

Samples were collected at day 6, 27 and 41 from the same three ECs utilized for cysteine oxidation investigations. The preparation of Hb-depleted soluble extracts and integral and cytoskeleton membrane extracts is extensively detailed in the Part 2, Chapter 2. Briefly, washed collected RBCs were submitted to hypotonic lysis, cytoplasm and membranes were separated by ultracentrifugation, soluble fraction was depleted in hemoglobin by IMAC, membranes were washed of any residual soluble components and membrane proteins were sequentially extracted by a non-ionic then an ionic detergent-containing buffer, solubilizing integral and cytoskeleton membrane proteins, respectively. The soluble Hb fraction was not investigated here.

Derivatization of carbonylated proteins

Five hundred µg of protein extract were submitted to protein carbonyl derivatization by incubation with 1 sample volume of a 10 mM Biotin-Hydrazide (BHZ) solution, during 1 hour at room temperature. The derivatization reaction was then stabilized through 40 minutes incubation with 2 sample volumes of a 30 mM CH_3BNNa solution on ice. Afterwards, excess of BHZ was removed by 3 concentration-dilution cycles (final dilution factor ≅ 1000), realized by centrifuge-driven filtration through 5000 MWCO-PES membranes.

Affinity-purification of derivatized proteins

Derivatized proteins were then purified using the biotin-avidin affinity. Samples were incubated with an excess of monomeric avidin-coated magnetic beads (Bioclone Inc, USA), during 1 hour at room temperature under slight agitation. The flow-through was collected, and beads were washed 3 times with 1x PBS. Then, captured proteins were eluted by incubating beads with 2 mM D-Biotin in PBS during 10 minutes, followed by another 10 minutes-incubation with 0.1 M Glycine, pH 2.8, both under agitation at room temperature.

1D-SDS-PAGE fractionation of purified proteins

Eluates were pooled, proteins were TCA-precipitated and acetone-washed. The entire pellets were resuspended in Laemmli buffer and loaded onto precast SDS-PAGE gels (Mini-PROTEAN

TGX Gels 4-15%, BIO-RAD, Hercules, USA). Unbound material (flow through, 20 µg) as well as 5 µL of BenchMark Protein Ladder was also loaded. Electrophoresis was performed in a Tris/Glycine/SDS buffer at constant 120 V for 5 minutes then constant 300 V until complete migration. Total proteins were finally revealed by Coomassie Blue staining.

LC-MS/MS identification of carbonylated proteins

Eluates migration lanes were cut in several pieces and some bands were also cut individually. The pre-analytics for LC-MS/MS identification is widely described in Chapter 1. Briefly, gel pieces were destained, proteins were submitted to reduction, alkylation and trypsin-driven digestion. Peptides were extracted, washed, and dried-stored prior to analysis. LC-MS/MS protein identifications were carried out as described in Chapter 1, except that no cysteine-labeling was considered as variable modification for database searching. In addition, no variable modification for specific detection of carbonylation was included, due to the high variation of existing modifications. Sites of carbonylation will thus not be assessed here, and carbonylated peptides will not be sequenced. As a consequence, the sequence coverage is inversely proportional to the amount of carbonylation.

At each storage point, only proteins that were identified in at least 2 out of the 3 biological replicates were considered as truly representative of the condition. List of proteins were submitted to GO annotation using Blast2GO®.

Results and discussion

Storage-related evolution of SDS-PAGE patterns of soluble and membrane carbonylome

One dimensional electrophoresis patterns of carbonylated proteins purification (eluates and flow through) from Hb-depleted soluble fraction and DDM-soluble membrane fraction of three ECs stored for 6, 27 and 41 days are presented in Figure 27. Focusing on soluble carbonylated proteins (Figure 27A), it is noteworthy that SDS-PAGE patterns at day 41 presented clear differences from days 6 and 27 samples, for each of the three ECs studied. In particular, a loss of content of more than 80 kDa was clearly observed. This is also, at a lower extent, the case for the unbound material (Figure 27B). This phenomenon inducing a loss of soluble high MW carbonylated content should thus be due to a storage-dependent mechanism. However, it does not seem concordant with quantitative analysis presented in Part 2, where a quantitative decrease of soluble carbonylated content was highlighted in the beginning of the storage period and not in the end. Some SDS-PAGE pattern differences were also detected at day 41 with the appearance of novel bands at 40 kDa and just below 20 kDa as well as a clear intensity decrease of the band just below 40 kDa.

Concerning DDM-soluble membrane carbonylated proteins, the SDS-PAGE patterns presented in Figure 27C do not reveal difference between storage duration. One can only note the appearance of a novel band at around 35 kDa, but only for the three samples from EC2. What should be noted is that the global patterns of carbonylated membrane proteins differed from a classic pattern of DDM-soluble RBCs membrane proteins (see Annex B), which much looks like the pattern of unbound membrane material, shown in Figure 27D. In particular, the highly abundant Band 3 protein (diffused band around 100 kDa) appeared very lowered in intensity in carbonylated protein samples (Figure 27C), suggesting that most copies of this protein should not suffer from carbonylation modification. Enrichment of altered Band 3 in microvesicles released during RBCs storage could probably explain this observation [24].

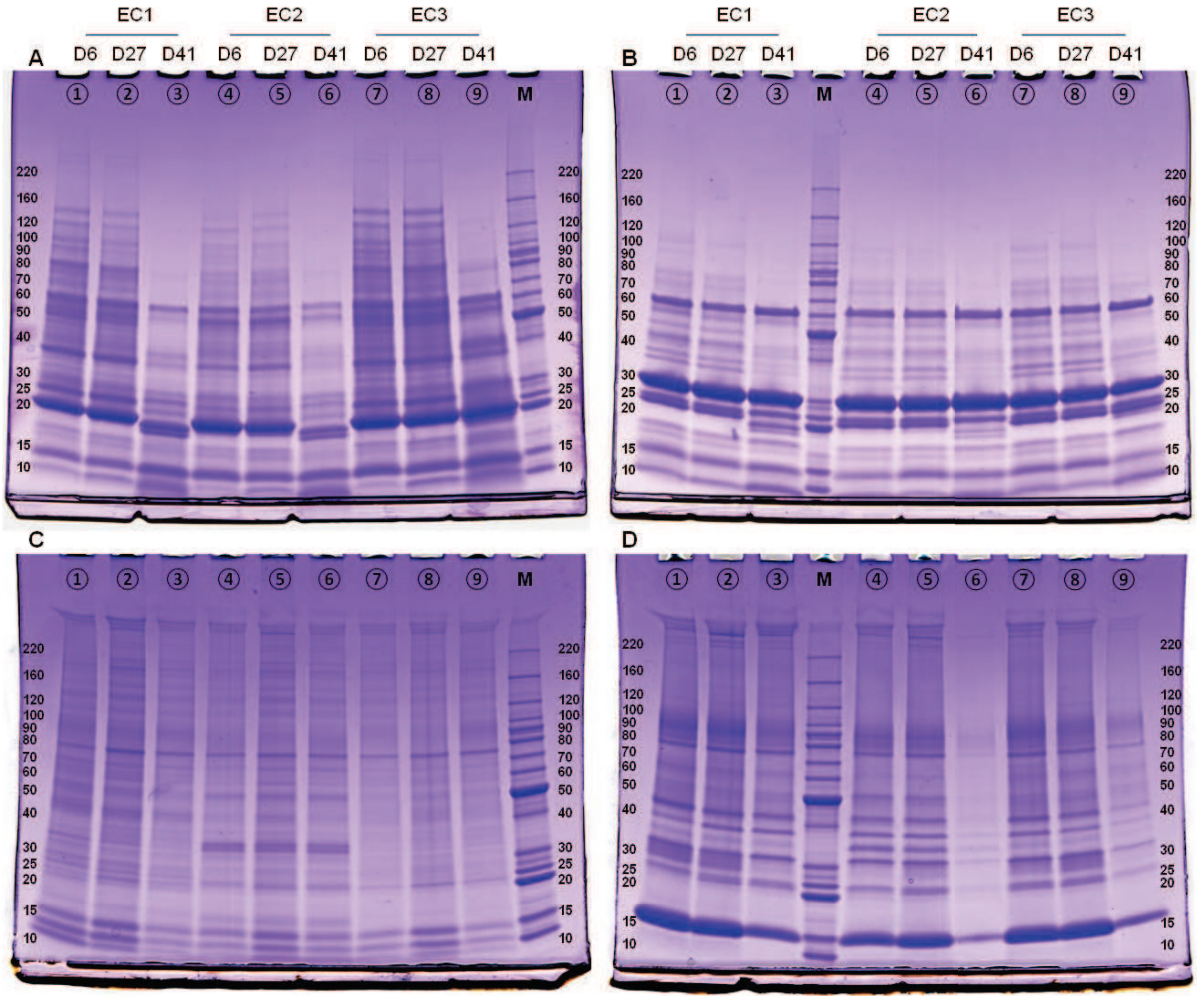


Figure 27: 1D electrophoresis patterns of the purification of carbonylated proteins in soluble and membrane extracts of stored RBCs. All purified carbonylated protein contents stemming from 500 µg of Hb-depleted soluble extracts (A) and DDM-soluble membrane extracts (C), as well as 20 µg of corresponding unbound proteins (B and D respectively) were submitted to SDS-PAGE on 4-15 % precast mini-gels and proteins were revealed by Coomassie Blue staining.

RBCs soluble storage-related carbonylome

Migration lanes of the 9 samples of soluble carbonylated proteins (3 ECs x 3 storage durations) were cut in 5 gel pieces so that bands were mostly equally distributed, avoiding overabundance issue. Proteins in each gel pieces were destained, trypsin-digested, and peptides were resuspended in LC-MS-compatible buffer for LC-MS/MS analyses. A total of 142 proteins were identified. Table 3 summarizes the identification parameters of each protein in a global analysis (merged LC-MS/MS data from all gel pieces). Proteins are sorted according to their accession number. Then for each protein, identification parameters are presented per storage duration.

For each storage duration, a protein was validated if identified in at least 2 out of the 3 biological replicates (3 ECs). As shown in the Venn diagram representation (see Figure 28), lots of identified proteins were common either between the 3 storage durations (28 %) or between days 6 and 27 (38 %). Only 56 proteins were identified at day 41. This low number seems obvious regarding SDS-PAGE pattern differences above-highlighted (see Figure 27A). Day 6 samples allowed the specific identification of 16 % of proteins, whereas days 27 and 41 samples revealed only 6 and 4 % of specific proteins, respectively. A few proteins (5 %) were commonly identified in days 27 and 41 samples, and 2 % of identified proteins appeared to be common to days 6 and 41 samples.

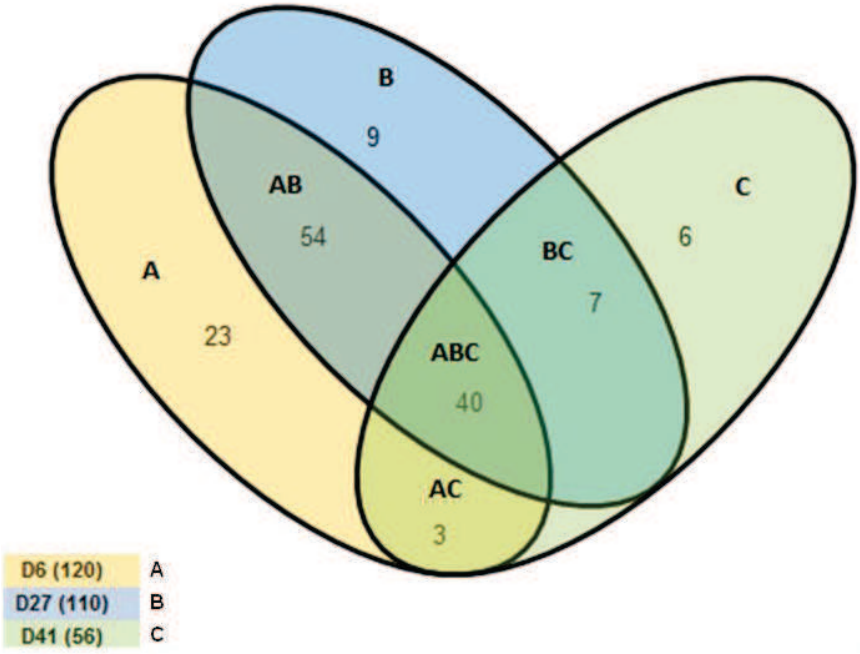


Figure 28: Venn diagram of cytosolic carbonylome during RBCs storage. 142 proteins were identified, of which 27.6 % present storage-time specificity. 28.3 % of identified proteins are found throughout the storage period. 83.4 % and 76.6 % of carbonylated proteins are detected at days 6 and 27 respectively, whereas there is a clear decrease in carbonylome content at day 41 (only 40.7 % of identified proteins).

Molecular functions engaged with identified soluble carbonylated proteins were investigated by GO annotation. Figure 29 shows the repartition of these molecular functions for the entire pool of identified proteins as well as for all proteins identified in each storage duration sample, and for the proteins that were specifically identified in each storage duration. The mean distribution

(Figure 29A) revealed a high part of binding and catalytic activities (46.4 and 44.7 %, respectively). Others molecular functions are antioxidant activity (3.4 %), enzyme regulation activity (1.7 %) and transporter activity (1.1 %). When looking at proteins identified without specificity for days 6, 27 and 41 samples (Figure 29B, C and D, respectively), this repartition appears quite similar to the mean one. There is however a tendency over the storage period of increasing part of the catalytic activity in disfavor of the binding activity. In addition, the proportion of annotated sequences related to antioxidant activity showed a 2-fold increase, from 2.6 % at day 6 to 5.2 % at day 41. This is interestingly concordant with the results of cysteine oxidation concerning the category of spots showing an increasing labeling signal ("up" group) where annotated sequences related to a peroxidase-antioxidant activity were twice as abundant as in the main distribution.

When dealing with carbonylated proteins specifically identified in the different storage durations (Figure 29E, F and G for days 6, 27 and 41, respectively), the repartition diagrams appeared very different compared to the main distribution (Figure 29A). The binding-related annotated sequences decreased from 48.9 % at day 6 to 36.8 % at day 41, with a main decrease in the first half of the storage (31.8 % at day 27). On the contrary, catalytic activity shows an increasing proportion from 24.4 % at day 6 to 36.8 % at day 41, with a main increase during the first half of the storage period too (36.4 % at day 27). As for antioxidant activity proportion, it was null at day 6, then increased to reach 13.6 % at day 27 and was finally decreased at 5.3 % at day 41. It thus seems that soluble antioxidant proteins undergo high carbonylation events during the first half of the storage period, then part of them are somehow processed making more difficult to purify them from soluble extracts of end-stored RBCs. When looking at oxidoreductase activity-related proteins (comprising the antioxidant ones), 15 proteins were identified as carbonylated at day 6, 18 proteins at day 27 and 12 proteins at day 41. Nine OR proteins were carbonylated throughout the storage period (6PGD, AL1A1, CATA, DCXR, LDHB, PRDX1, PRDX2, PRDX6 and THIO), 4 OR proteins were carbonylated at days 6 and 27 (A16A1, Al9A1, CBR1 and G3P), but were not identified at day 41, and 2 OR proteins were not found carbonylated at day 6 but were identified at days 27 and 41 (BIEA and LDHA).

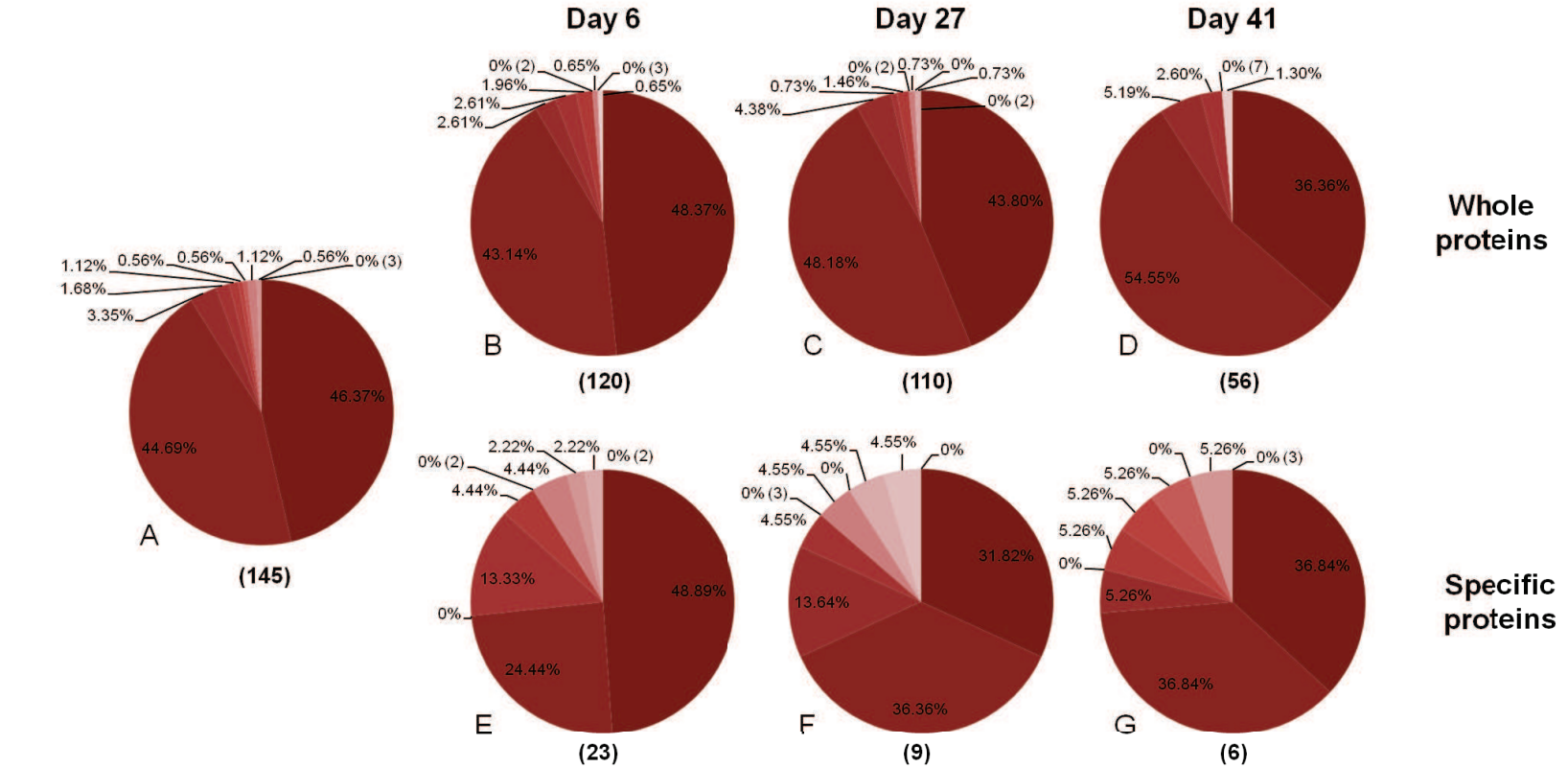


Figure 29: Repartition of molecular functions corresponding to cytosolic carbonylated proteins during RBCs storage. Diagram A represents the repartition of molecular functions for all identified soluble carbonylated proteins. Diagrams B to G represent the repartitions at days 6, 27 and 41, either for all proteins identified at each day (B, C and D, respectively) or for proteins that were specifically identified at each day (E, F and G, respectively). The bracketed numbers following some "0 %"-labels indicate that number of following unrepresented molecular functions, according to the ranking in the legend. Under each diagram figured the number of identified proteins. The color shading in the legend should be read from left to right then top to down.

Table 3: Proteomic identification of the RBC soluble carbonylome during storage

Name	AC #	Global analysis			Storage-time analysis			
		Seq. cov. (%)	Peptide #	ID score	Sample	Seq. cov. (%)	Peptide #	ID score
14-3-3 protein epsilon	**1433E_HUMAN**	57.70	13	651.49	D6 D27 D41	34.10 25.90 55.70	6 5 12	284.76 237.92 617.39
Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	**2AAA_HUMAN**	25.80	10	470.26	D6 D27	21.40 24.40	10 9	412.79 373.76
6-phosphogluconate dehydrogenase	**6PGD_HUMAN**	42.00	17	797.82	D6 D27 D41	32.50 33.10 19.90	13 11 7	581.63 566.77 385.31
Aldehyde dehydrogenase family 16 member A1	**A16A1_HUMAN**	17.80	12	579.48	D6 D27	17.80 15.50	12 10	497.92 406.35
Abhydrolase domain-containing protein 14B	**ABHEB_HUMAN**	14.30	3	109.01	D6 D27	14.30 9.50	3 2	99.27 86.71
ATP-citrate synthase	**ACLY_HUMAN**	12.30	11	420.55	D6	8.40	8	293.01
Acylamino-acid-releasing enzyme	**ACPH_HUMAN**	18.70	12	663.99	D6 D27 D41	16.40 16.70 13.90	10 12 10	517.78 565.19 519.68
Retinal dehydrogenase 1	**AL1A1_HUMAN**	37.30	19	930.77	D6 D27 D41	37.30 35.10 31.70	18 17 17	815.64 806.84 821.79
4-trimethylaminobutyraldehyde dehydrogenase	**AL9A1_HUMAN**	15.60	5	279.01	D6 D27	12.80 14.00	4 5	211.46 248.26
Serum albumin	**ALBU_HUMAN**	19.00	10	410.81	D6 D27 D41	5.40 11.80 16.40	3 4 9	115.34 194.29 357.35
Fructose-bisphosphate aldolase A	**ALDOA_HUMAN**	54.40	18	1050.79	D6 D27 D41	46.20 47.80 52.70	12 12 17	624.48 641.48 944.57
Protein arginine N-methyltransferase 5	**ANM5_HUMAN**	12.60	6	292.71	D6 D27	12.60 6.90	6 4	281.17 174.86
ADP-ribosylation factor 4	**ARF4_HUMAN**	19.30	4	187.96	D6	9.40	2	89.97

Name	AC #	Global analysis			Storage-time analysis			
		Seq. cov. (%)	Peptide #	ID score	Sample	Seq. cov. (%)	Peptide #	ID score
Arginase-1	**ARGI1_HUMAN**	26.40	7	286.46	D6 D27 D41	17.40 21.10 19.90	4 4 7	192.28 194.16 248.49
Biliverdin reductase A	**BIEA_HUMAN**	37.50	9	379.83	D27 D41	27.40 37.50	4 9	225.00 329.85
Flavin reductase (NADPH)	**BLVRB_HUMAN**	47.10	7	496.15	D6 D27 D41	35.00 30.60 47.10	4 4 7	309.58 290.85 492.05
Carbonic anhydrase 1	**CAH1_HUMAN**	61.30	12	674.19	D6 D27 D41	26.80 18.80 61.30	5 4 12	309.76 175.59 674.19
Carbonic anhydrase 3	**CAH3_HUMAN**	26.90	4	226.41	D41	26.90	4	226.41
Calpain-1 catalytic subunit	**CAN1_HUMAN**	21.10	14	650.39	D6 D27	19.30 18.80	14 13	590.42 558.77
Cullin-associated NEDD8-dissociated protein 1	**CAND1_HUMAN**	33.00	32	1463.17	D6 D27	27.50 31.10	26 28	1131.33 1284.39
F-actin-capping protein subunit beta	**CAPZB_HUMAN**	14.40	3	178.70	D6 D27	6.90 14.40	2 3	76.09 176.33
Catalase	**CATA_HUMAN**	68.10	33	1868.26	D6 D27 D41	65.70 62.80 65.80	30 28 31	1563.58 1612.91 1639.67
F-actin-capping subunit alpha-1	**CAZA1_HUMAN**	11.90	2	125.34	D6 D27	11.90 9.80	2 2	108.90 101.39
Carbonyl reductase [NADPH] 1	**CBR1_HUMAN**	23.50	5	240.24	D6 D27	23.50 11.20	5 2	240.24 143.51
Cell division control protein 42 homolog	**CDC42_HUMAN**	19.90	3	148.74	D6 D27	19.90 19.90	3 3	135.88 141.13
Calcium-regulated heat stable protein 1	**CHSP1_HUMAN**	28.60	4	118.69	D6	28.60	4	116.40
Cofilin-1	**COF1_HUMAN**	60.80	10	477.86	D6 D27	60.80 57.20	10 9	406.98 439.90
COP9 signalosome complex subunit 1	**CSN1_HUMAN**	7.50	2	119.24	D6	5.50	2	100.11
COP9 signalosome complex subunit 3	**CSN3_HUMAN**	12.80	5	216.05	D6 D27	12.80 10.90	4 4	205.83 162.57

Name	AC #	Global analysis			Storage-time analysis			
		Seq. cov. (%)	Peptide #	ID score	Sample	Seq. cov. (%)	Peptide #	ID score
COP9 signalosome complex subunit 4	**CSN4_HUMAN**	26.60	8	387.07	D6 D27	25.40 20.00	8 5	362.27 242.09
COP9 signalosome complex subunit 5	**CSN5_HUMAN**	7.50	3	121.47	D27	7.50	3	114.30
COP9 signalosome complex subunit 6	**CSN6_HUMAN**	16.20	5	166.21	D6	16.20	5	148.45
Uncharacterized protein C22orf25	**CV025_HUMAN**	8.30	3	114.51	D41	5.80	2	76.21
Uroporphyrinogen decarboxylase	**DCUP_HUMAN**	10.60	3	116.48	D41	10.60	3	116.48
L-xylulose reductase	**DCXR_HUMAN**	48.00	10	431.49	D6 D27 D41	42.60 48.00 20.50	8 9 4	318.13 399.31 172.03
DNA damage-binding protein 1	**DDB1_HUMAN**	12.40	12	507.28	D6	11.00	9	405.29
Protein DDI1 homolog 2	**DDI2_HUMAN**	38.30	9	472.91	D6 D27	29.30 36.10	8 8	389.61 412.38
Putative deoxyribose-phosphate aldolase	**DEOC_HUMAN**	23.90	5	232.30	D6 D27	20.80 13.20	4 3	207.25 154.77
Bifunctional ATP-dependent dihydroxyacetone kinase/FAD-AMP lyase (cyclizing)	**DHAK_HUMAN**	21.90	9	364.53	D6 D27	19.50 17.90	6 7	306.39 256.99
Alpha-enolase	**ENOA_HUMAN**	54.40	20	913.09	D6 D27 D41	53.70 50.90 42.20	15 18 16	729.81 772.31 723.96
S-formylglutathione hydrolase	**ESTD_HUMAN**	55.30	10	418.68	D6 D27 D41	34.80 35.10 39.40	6 7 9	302.85 287.69 320.83
Hsc70-interacting protein	**F10A1_HUMAN**	29.80	9	547.19	D6 D27	29.80 29.80	9 9	486.41 482.58
Fatty acid synthase	**FAS_HUMAN**	5.50	7	425.35	D6	4.00	5	270.74
F-box only protein 7	**FBX7_HUMAN**	10.70	5	205.33	D27	3.80	2	92.03
GDP-L-fucose synthase	**FCL_HUMAN**	11.50	3	142.26	D6	8.10	2	90.75
Fumarate hydratase, mitochondrial	**FUMH_HUMAN**	19.20	7	293.41	D6 D27	11.60 14.70	5 6	208.71 228.84
Glyceraldehyde-3-phosphate dehydrogenase	**G3P_HUMAN**	54.30	17	898.23	D6 D27	54.00 50.70	16 14	786.94 764.75

Name	AC #	Global analysis			Storage-time analysis			
		Seq. cov. (%)	Peptide #	ID score	Sample	Seq. cov. (%)	Peptide #	ID score
Hydroxyacylglutathione hydrolase, mitochondrial	**GLO2_HUMAN**	29.90	8	303.51	D6 D27 D41	22.40 26.30 22.40	5 7 6	179.42 255.36 192.17
Glutaredoxin-1	**GLRX1_HUMAN**	31.10	3	175.17	D27	31.10	3	173.48
Glutathione peroxidase 1	**GPX1_HUMAN**	40.40	8	249.20	D27	40.40	7	220.40
Glutamate-cysteine ligase catalytic subunit	**GSH1_HUMAN**	20.40	11	481.65	D6 D27	12.90 19.50	7 10	275.51 402.32
Glutathione S-transferase omega-1	**GSTO1_HUMAN**	38.60	9	425.76	D6 D27 D41	27.00 24.10 38.60	6 7 8	245.39 219.55 418.69
Hemoglobin subunit alpha	**HBA_HUMAN**	92.30	11	558.14	D6 D27 D41	91.50 72.50 91.50	10 9 10	493.56 423.97 484.47
Hemoglobin subunit beta	**HBB_HUMAN**	95.20	14	811.18	D6 D27 D41	95.20 95.20 95.20	13 13 14	733.45 686.97 730.77
Hemoglobin subunit delta	**HBD_HUMAN**	54.40	9	503.32	D41	54.40	9	448.95
Hemoglobin subunit gamma-2	**HBG2_HUMAN**	22.40	2	148.15	D6	15.60	2	106.45
Delta-aminolevulinic acid dehydratase	**HEM2_HUMAN**	52.10	18	984.52	D6 D27 D41	46.10 52.10 50.60	13 15 18	645.32 719.53 925.51
Hypoxanthine-guanine phosphoribosyltransferase	**HPRT_HUMAN**	47.20	8	408.38	D6 D27 D41	44.00 42.71 35.30	7 8 7	331.99 361.47 309.27
Heat shock protein HSP 90-alpha	**HS90A_HUMAN**	24.60	15	713.38	D6 D27	18.30 19.30	12 11	486.53 539.38
Heat shock 70 kDa protein 1A/1B	**HSP71_HUMAN**	19.80	9	456.97	D27	10.60	6	268.93
Heat shock 70 kDa protein 4	**HSP74_HUMAN**	4.60	2	121.21	D6 D27	4.60 2.30	1 2	100.21 86.91
Heat shock cognate 71 kDa protein	**HSP7C_HUMAN**	30.50	15	706.84	D6 D27	24.00 18.10	14 11	573.77 447.68
Eukaryotic initiation factor 4A-I	**IF4A1_HUMAN**	19.00	7	229.10	D6	19.00	7	229.10

Name	AC #	Global analysis			Storage-time analysis			
		Seq. cov. (%)	Peptide #	ID score	Sample	Seq. cov. (%)	Peptide #	ID score
Importin subunit beta-1	**IMB1_HUMAN**	14.40	9	427.40	D6	13.40	9	400.32
					D27	9.70	5	243.47
Importin-5	**IPO5_HUMAN**	4.50	3	150.83	D6	1.70	2	74.50
					D27	4.40	3	134.56
Importin-7	**IPO7_HUMAN**	2.80	2	146.04	D6	2.00	2	127.07
Adenylate kinase isoenzyme 1	**KAD1_HUMAN**	73.70	10	625.80	D6	69.10	10	545.68
					D27	60.30	9	459.35
					D41	33.00	6	276.42
Pyruvate kinase isozymes R/L	**KPYR_HUMAN**	50.30	22	1033.64	D6	40.40	18	820.35
					D27	49.30	21	911.79
L-lactate dehydrogenase A chain	**LDHA_HUMAN**	29.20	10	478.44	D27	21.40	7	317.51
					D41	16.90	4	284.27
L-lactate dehydrogenase B chain	**LDHB_HUMAN**	40.40	13	682.36	D6	40.40	13	662.69
					D27	37.70	10	556.25
					D41	32.90	9	513.09
Lactoylglutathione lyase	**LGUL_HUMAN**	52.20	9	392.86	D27	17.40	3	142.64
					D41	51.10	9	374.50
Leukotriene A-4 hydrolase	**LKHA4_HUMAN**	16.00	6	264.20	D6	10.00	5	191.50
					D27	11.00	4	197.53
Latexin	**LXN_HUMAN**	19.40	3	125.41	D6	19.40	3	125.41
Moesin	**MOES_HUMAN**	34.00	21	1043.78	D6	13.70	9	462.42
					D27	21.00	12	513.57
					D41	34.00	21	939.83
Nucleoside diphophate kinase A	**NDKA_HUMAN**	69.10	12	682.96	D6	65.80	9	479.82
					D27	69.10	9	460.01
					D41	65.80	12	614.87
Nucleoside diphosphate kinase B	**NDKB_HUMAN**	67.10	11	510.04	D6	50.00	8	355.91
					D41	67.10	11	456.28
NIF3-like protein 1	**NIF3L_HUMAN**	13.80	4	193.54	D6	7.40	3	130.53
					D27	13.80	4	169.69
Obg-like ATPase 1	**OLA1_HUMAN**	22.00	5	355.23	D6	14.60	4	284.28
					D27	17.90	5	293.67
					D41	10.60	4	216.92

Name	AC #	Global analysis			Storage-time analysis			
		Seq. cov. (%)	Peptide #	ID score	Sample	Seq. cov. (%)	Peptide #	ID score
Platelet-activating factor acetylhydrolase IB subunit beta	PA1B2_HUMAN	8.30	1	76.33	D6 D41	8.30 8.30	1 1 1	67.42 64.14
Platelet-activating factor acetylhydrolase IB subunit gamma	PA1B3_HUMAN	29.00	5	277.82	D6 D27 D41	17.70 29.00 21.20	5 5 4	202.24 265.59 206.75
Proliferation-associated protein 2G4	PA2G4_HUMAN	21.60	7	283.54	D6 D27	14.50 21.60	6 7	219.95 269.71
Protein DJ-1	PARK7_HUMAN	30.20	6	250.19	D6 D27 D41	30.20 30.20 30.20	6 6 6	231.93 234.54 216.27
Programmed cell death 6-interacting protein	PDC6I_HUMAN	19.80	14	575.81	D6	17.30	12	438.01
Phosphoglucomutase-2	PGM2_HUMAN	18.50	8	412.83	D6 D27	14.40 13.40	6 6	309.83 302.99
Glucose 1,6-bisphosphate synthase	PGM2L_HUMAN	22.20	12	453.29	D6 D27	13.70 20.70	8 10	303.56 400.90
Protein –L-isoaspartate(D-aspartate) O-methyltransferase	PIMT_HUMAN	39.20	6	279.54	D6 D27	19.80 39.20	3 6	169.70 249.49
Nicotinate phosphoribosyltransferase	PNCB_HUMAN	22.70	9	515.29	D6 D27	19.50 20.30	8 8	385.87 450.60
Purine nucleoside phosphorylase	PNPH_HUMAN	62.60	18	772.79	D27 D41	14.90 62.60	3 18	139.63 772.79
Low molecular weight phosphotyrosine protein phosphatase	PPAC_HUMAN	44.30	8	346.24	D6 D27 D41	36.70 44.30 43.70	6 7 6	301.74 306.30 238.45
Peptidyl-prolyl cis-trans isomerase A	PPIA_HUMAN	31.50	5	302.80	D6 D27	31.50 25.50	4 4	269.59 241.71
Peroxiredoxin-1	PRDX1_HUMAN	81.40	14	742.19	D6 D27 D41	54.80 81.40 54.80	10 12 13	488.24 639.82 627.52
Peroxiredoxin-2	PRDX2_HUMAN	65.70	16	978.05	D6 D27 D41	63.10 63.60 60.10	16 16 14	857.13 881.29 880.42

Name	AC #	Global analysis			Storage-time analysis			
		Seq. cov. (%)	Peptide #	ID score	Sample	Seq. cov. (%)	Peptide #	ID score
Peroxiredoxin-6	**PRDX6_HUMAN**	79.50	15	730.32	D6 D27 D41	79.50 76.30 51.30	14 15 10	582.09 677.10 371.88
Ribose-phosphate pyrophosphokinase 1	**PRPS1_HUMAN**	26.10	8	289.67	D6 D27	26.10 26.10	8 8	246.06 260.43
Puromycin-sensitive aminopeptidase	**PSA_HUMAN**	16.80	14	543.77	D6 D27	12.20 16.80	9 14	334.76 519.48
Proteasome subunit alpha type-3	**PSA3_HUMAN**	14.90	4	162.58	D41	14.90	3	155.84
Proteasome subunit alpha type-6	**PSA6_HUMAN**	13.80	2	120.73	D27 D41	10.20 5.30	1 1	93.73 82.25
26S proteasome non-ATPase regulatory subunit 7	**PSD7_HUMAN**	7.40	2	79.83	D6	7.40	2	79.83
26S proteasome non-ATPase regulatory subunit 2	**PSMD2_HUMAN**	26.20	17	797.08	D6 D27	25.10 20.20	15 12	709.68 533.21
26S proteasome non-ATPase regulatory subunit 3	**PSMD3_HUMAN**	10.90	6	264.00	D6	8.40	5	189.71
26S proteasome non-ATPase regulatory subunit 6	**PSMD6_HUMAN**	24.70	9	356.83	D6	24.70	9	349.86
Proteasome activator complex subunit 1	**PSME1_HUMAN**	36.90	8	330.39	D6	23.70	6	206.29
Serine/threonine-protein phosphatase 2A activator	**PTPA_HUMAN**	6.40	2	91.42	D6 D41	6.40 4.70	1 1	79.20 76.20
Putative peptidyl-tRNA hydrolase PTRHD1	**PTRD1_HUMAN**	12.10	2	76.98	D6	12.10	2	76.98
Phosphoribosylformylglycinamidine synthase	**PUR4_HUMAN**	19.80	19	941.55	D6 D27	17.60 16.00	17 14	717.83 758.12
Multifunctional protein ADE2	**PUR6_HUMAN**	33.90	9	413.33	D6 D27	24.50 23.80	8 7	322.69 308.76
Adenylosuccinate lyase	**PUR8_HUMAN**	15.90	7	341.88	D6 D27	13.00 13.60	5 6	301.04 281.24
GTP-binding nuclear protein Ran	**RAN_HUMAN**	45.40	10	482.70	D6 D27 D41	42.60 45.40 25.00	8 9 5	406.09 427.30 186.05
Ran-specific GTPase-activating protein	**RANG_HUMAN**	18.90	3	180.78	D6 D27	15.40 18.90	3 3	149.44 180.78
Ribonuclease inhibitor	**RINI_HUMAN**	41.20	12	688.01	D6 D27	31.70 27.10	9 8	552.09 402.63

Name	AC #	Global analysis			Storage-time analysis			
		Seq. cov. (%)	Peptide #	ID score	Sample	Seq. cov. (%)	Peptide #	ID score
Ubiquitin-60S ribosomal protein L40	**RL40_HUMAN**	46.10	3	190.59	D27	46.10	3	190.59
E3 ubiquitin-protein ligase RNF123	**RN123_HUMAN**	14.50	16	714.62	D6	13.30	16	634.00
					D27	10.70	11	433.54
Ribose-5-phosphate isomerase	**RPIA_HUMAN**	19.60	7	344.65	D27	17.00	5	278.14
					D41	19.30	6	245.10
Ubiquitin-40S ribosomal protein S27a	**RS27A_HUMAN**	42.30	4	219.64	D6	42.30	4	186.48
RuvB-like 1	**RUVB1_HUMAN**	12.50	2	112.15	D6	12.50	2	112.15
RuvB-like 2	**RUVB2_HUMAN**	13.40	6	278.11	D6	13.40	6	266.66
Protein S100-A4	**S10A4_HUMAN**	45.50	9	410.24	D6	41.60	7	316.00
					D27	45.50	5	267.17
					D41	35.60	5	253.87
Adenosylhomocysteinase	**SAHH_HUMAN**	36.10	14	716.56	D6	22.70	8	398.57
					D27	26.60	9	433.54
					D41	33.80	13	636.15
Selenium-binding protein 1	**SBP1_HUMAN**	81.10	31	1742.78	D6	67.60	23	1271.27
					D27	67.60	24	1252.25
					D41	76.10	31	1614.49
S-phase kinase-associated protein 1	**SKP1_HUMAN**	40.50	4	231.78	D6	36.80	4	199.40
					D27	36.20	4	171.05
Superoxide dismutase [Cu-Zn]	**SODC_HUMAN**	51.30	5	198.11	D27	51.30	4	191.80
Sorcin	**SORCN_HUMAN**	30.30	6	230.21	D6	26.30	5	192.24
					D27	30.30	6	215.27
					D41	30.30	3	161.48
Spectrin alpha chain, erythrocyte	**SPTA1_HUMAN**	5.90	9	386.39	D6	4.90	7	310.11
Stress-induced-phosphoprotein 1	**STIP1_HUMAN**	32.40	14	617.45	D6	28.90	14	562.17
					D27	22.30	12	466.99
Alpha-synuclein	**SYUA_HUMAN**	60.00	8	400.83	D6	60.00	6	252.24
					D27	53.60	7	352.51
Transaldolase	**TALDO_HUMAN**	15.10	3	149.44	D41	12.50	3	126.34
T-complex protein 1 subunit gamma	**TCPG_HUMAN**	15.00	7	353.43	D6	9.20	4	205.57
					D27	15.00	7	329.75

Name	AC #	Global analysis			Storage-time analysis			
		Seq. cov. (%)	Peptide #	ID score	Sample	Seq. cov. (%)	Peptide #	ID score
T-complex protein 1 subunit theta	**TCPQ_HUMAN**	27.90	14	735.76	D6 D27 D41	27.90 19.70 6.60	14 11 4	689.65 580.68 145.86
Transitional endoplasmic reticulum ATPase	**TERA_HUMAN**	42.70	28	1322.44	D6 D27 D41	41.70 34.00 22.00	26 16 12	1188.87 807.71 476.62
Protein-glutamine gamma-glutamyltransferase 2	**TGM2_HUMAN**	35.10	21	1123.36	D6 D27 D41	33.80 29.90 8.00	20 16 5	1024.04 886.44 181.90
Acetyl-CoA acetyltransferase, cytosolic	**THIC_HUMAN**	17.40	5	236.66	D27	13.40	4	171.22
Thioredoxin	**THIO_HUMAN**	42.90	4	250.57	D6 D27 D41	41.00 31.40 21.00	3 4 2	190.28 201.49 146.47
3-mercaptopyruvate sulfurtransferase	**THTM_HUMAN**	17.20	4	144.90	D6	17.20	4	139.72
Transketolase	**TKT_HUMAN**	10.60	4	211.78	D6 D27 D41	5.60 10.60 6.10	3 4 3	135.33 197.02 133.75
Tripeptidyl-peptidase 2	**TPP2_HUMAN**	7.50	8	314.51	D6 D27	4.70 7.50	5 8	198.31 295.65
Ubiquitin-conjugating enzyme E2 variant 1	**UB2V1_HUMAN**	22.40	4	165.46	D27 D41	22.40 22.40	4 4	152.82 143.35
Ubiquitin-like modifier-activating enzyme 1	**UBA1_HUMAN**	9.50	8	361.72	D6 D27	2.90 4.00	2 4	116.20 170.94
Ubiquitin-associated domain-containing protein 1	**UBAC1_HUMAN**	21.00	7	312.40	D6 D27	18.00 7.40	6 3	273.68 143.00
Ubiquitin carboxyl-terminal hydrolase 14	**UBP14_HUMAN**	24.90	9	524.01	D6 D27	21.30 24.90	7 8	444.89 448.73
Ubiquitin carboxyl-terminal hydrolase 5	**UBP5_HUMAN**	27.70	21	831.08	D6 D27	21.60 27.50	16 18	618.82 680.76
V-type proton ATPase catalytic subunit A	**VATA_HUMAN**	18.00	9	148.58	D6 D27	13.80 12.20	7 6	348.96 221.57

Name	AC #	Global analysis			Storage-time analysis			
		Seq. cov. (%)	Peptide #	ID score	Sample	Seq. cov. (%)	Peptide #	ID score
Exportin-7	**XPO7_HUMAN**	24.70	23	935.84	D6 D27	23.60 20.20	20 16	848.80 729.88

RBC membrane storage-related carbonylome

Migrations lanes of the 9 samples of DDM-soluble membrane carbonylated proteins were cut in 6 gel pieces and processed as described above. A total of 20 carbonylated proteins were identified with the same validation criteria as before. Table 4 summarizes the global identification parameters (merged LC-MS/MS data from all gel pieces) of proteins sorted by accession number, as well as in a storage-duration specific manner. As shown in Figure 30, most of the identified DDM-soluble membrane carbonylated proteins (55 %) were common to the 3 investigated storage durations. Only 2 proteins (10 %) were specifically identified in day 6 samples, and 4 proteins (20 %) were specific to day 27 samples. No protein was specific to end-stored samples, but the carbonylation of 2 proteins (10 %) seemed to be related to the second half of the storage period, being commonly identified in days 27 and 41 samples.

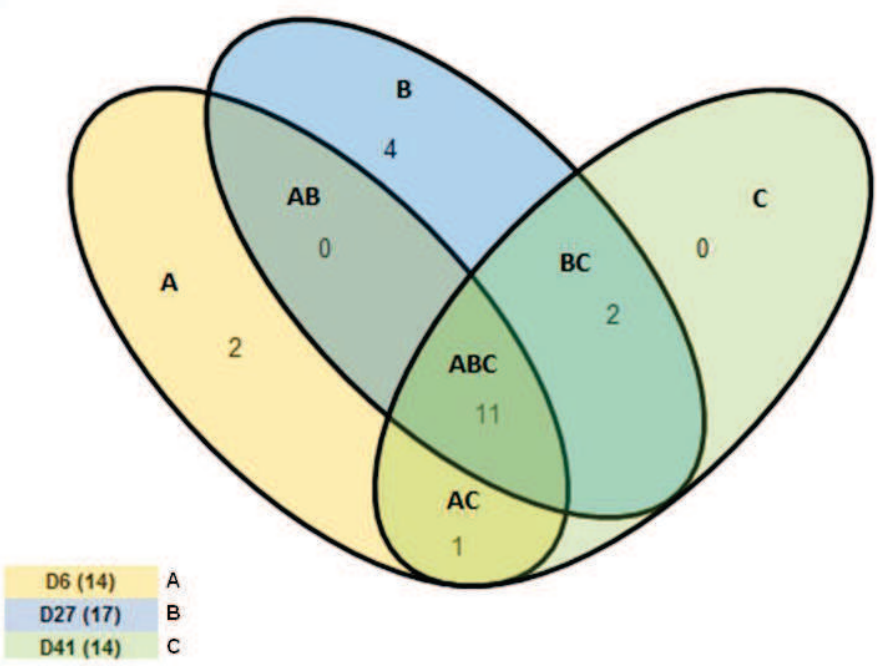


Figure 30: Venn diagram of integral membrane carbonylome during RBCs storage. Twenty proteins were identified as carbonylated proteins during the storage period of RBCs. More than the half (55 %) were common between the 3 investigated storage periods.

Through the 20 identified membrane carbonylated proteins, it appears that the more

represented molecular functions are binding activity (39.6 %), catalytic activity (18.8 %), antioxidant activity (8.3 %), transporter activity (12.5 %) and structural molecule activity (14.6 %), as shown in Figure 31A. If the 3 former ones are highly represented commonly with investigations on soluble proteins (both cysteine oxidation and protein carbonylation), the proportions taken by two latters seems obviously more specific to membrane proteins. As mentioned above, a very few proteins were specifically identified in a given storage duration. Interpreting the molecular functions distribution in storage time-specific membrane carbonylomes (Figure 31E and F for days 6 and 27 samples, respectively) seems thus not appropriate. Following descriptions will thus concern all identified proteins at each storage duration, as shown in Figure 31B, C and D. Along the storage period, it can be noted a slight increase in the proportion of catalytic activity-related annotated sequences (from 16.7 % at day 6 to 20.7% at day 41) and a concomitant slight decrease in the proportion of the transporter activity (from 16.7 % at day 6 to 13.8 % at day 41). The proportion of annotations related to structural molecule activity (cytoskeleton components) fluctuated between 20 % at day 6, 15.8 % at day 27 and 17.2 % at day 41. The loss of membrane-bounded carbonylated cytoskeletal proteins can be explained by the dissociation of cytoskeleton from membranes under ATP depletion conditions [25, 26], such as occurring during the EC storage. It is noteworthy that cytoskeleton oxidation increase during RBC storage, as shown in Part 2 and by others [27], and thiol oxidation of cytoskeleton was linked to the microvesiculation process [28, 29]. Morever, this membrane loss is thought to be a protective mechanism, allowing the elimination of dimerized Band 3, that is the RBC immunological clearance signal [30].

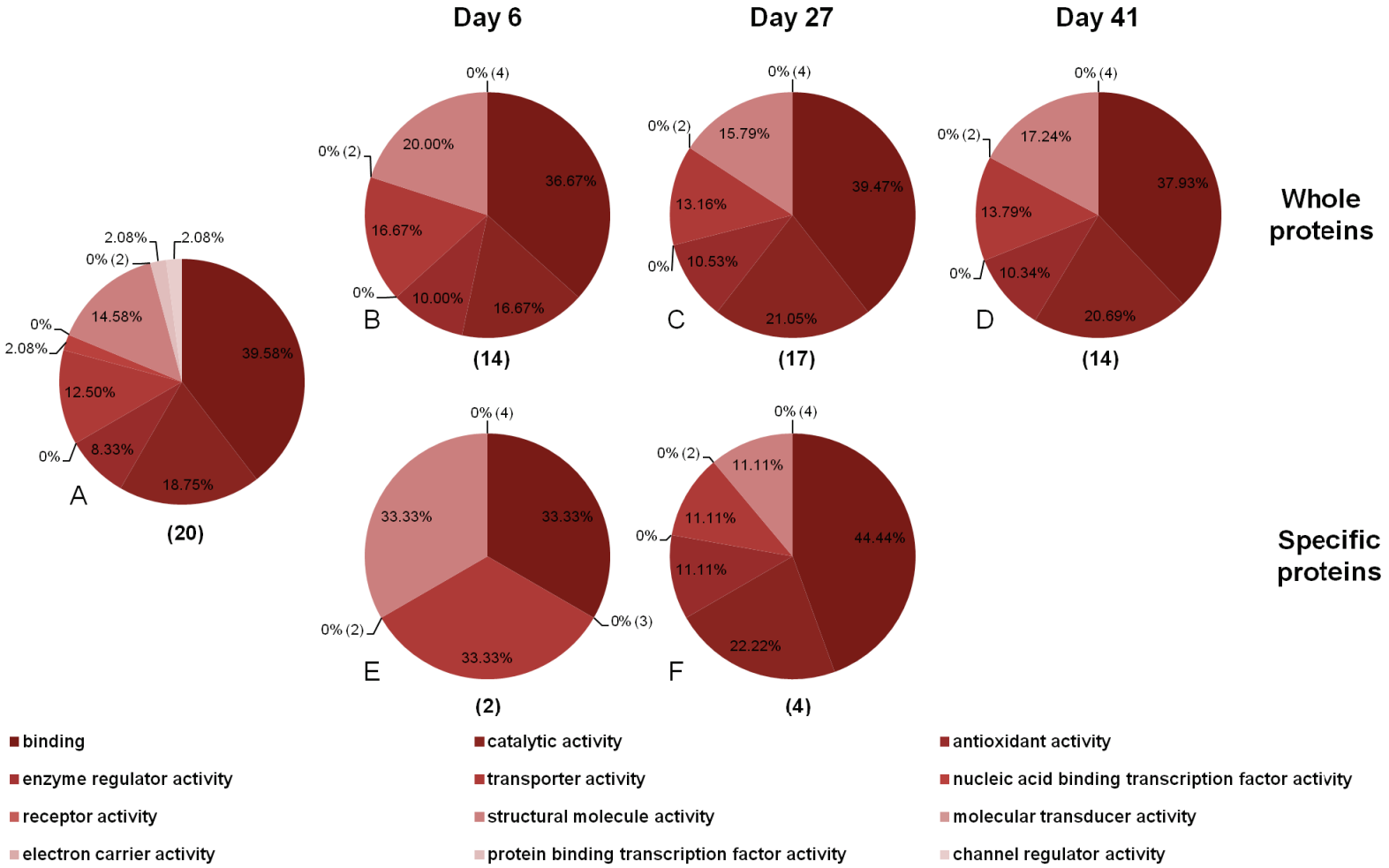


Figure 31: Repartition of molecular functions corresponding to integral carbonylated membrane proteins during RBCs storage. Diagram A represents the repartition of molecular functions for all identified membrane carbonylated proteins. Diagrams B to F represent the repartitions at days 6, 27 and 41, either for all proteins identified at each day (B, C and D, respectively) or for proteins that were specifically identified at each day (E and F, respectively). Note that no proteins were specifically identified for the day 41 condition. The bracketed numbers following some "0 %"-labels indicate that number of following unrepresented molecular functions, according to the ranking in the legend. Under each diagram figured the number of identified proteins. The color shading in the legend should be read from left to right then top to down.

Table 4: Proteomic identification of the RBC membrane carbonylome during RBCs storage

Name	AC #	Global analysis			Storage-time analysis			
		Seq. cov. (%)	Peptide #	ID score	Sample	Seq. cov. (%)	Peptide #	ID score
Protein 4.1	41_HUMAN	28.70	17	969.48	D6	20.80	12	645.97
					D27	22.50	13	767.61
					D41	17.60	13	697.80
ATP-citrate synthase	ACLY_HUMAN	6.70	3	215.50	D27	6.70	3	185.11
Actin, cytoplasmic 1	ACTB_HUMAN	16.80	4	175.22	D6	9.90	3	105.81
Alpha-adducin	ADDA_HUMAN	4.90	3	123.17	D27	4.90	3	123.17
Ankyrin-1	ANK1_HUMAN	29.30	40	2292.69	D6	19.50	24	1279.31
					D27	27.60	38	2135.72
					D41	17.30	25	1358.86
Band 3 anion transport protein	B3AT_HUMAN	39.30	28	1608.26	D6	32.20	25	1246.24
					D27	32.70	21	1343.07
					D41	35.80	22	1313.26
55 kDa erythrocyte membrane protein	EM55_HUMAN	8.80	4	180.34	D27	8.80	2	159.05
					D41	8.80	4	156.00
Erythrocyte membrane protein band 4.2	EPB42_HUMAN	46.90	25	1334.49	D6	21.60	11	550.88
					D27	46.90	25	1301.76
					D41	32.40	17	790.66
Glyceraldehyde-3-phosphate dehydrogenase	G3P_HUMAN	31.60	7	428.62	D6	17.00	4	276.99
					D27	19.40	5	291.86
					D41	31.60	7	359.51
Golgi-associated plant pathogenesis-related protein	GAPR1_HUMAN	16.90	2	137.13	D27	16.90	2	114.02
					D41	16.90	2	133.76
Solute carrier family 2, facilitated glucose transporter member 1	GTR1_HUMAN	8.10	5	288.42	D6	8.10	5	288.28
					D27	8.10	5	253.84
					D41	7.90	4	238.44
Hemoglobin subunit alpha	HBA_HUMAN	68.30	7	389.44	D6	47.90	7	334.86
					D27	47.90	6	348.51
					D41	68.30	5	313.71
Hemoglobin subunit beta	HBB_HUMAN	73.50	9	535.42	D6	73.50	9	459.41
					D27	73.50	8	460.18
					D41	73.50	8	490.24

Name	AC #	Global analysis			Storage-time analysis			
		Seq. cov. (%)	Peptide #	ID score	Sample	Seq. cov. (%)	Peptide #	ID score
Hemoglobin subunit delta	**HBD_HUMAN**	67.30	8	353.12	D27	54.40	6	294.03
Peroxiredoxin-2	**PRDX2_HUMAN**	35.40	6	332.18	D6	30.80	6	262.22
					D27	30.80	6	249.53
					D41	35.40	6	308.21
Blood group Rh(D) polypeptide	**RHD_HUMAN**	4.10	2	82.73	D6	4.10	2	75.25
Spectrin alpha chain, erythrocyte	**SPTA1_HUMAN**	43.00	84	4166.26	D6	25.30	47	2065.63
					D27	38.90	77	3956.26
					D41	21.30	43	1932.65
Spectrin beta chain, erythrocyte	**SPTB1_HUMAN**	27.70	47	2591.54	D6	16.40	30	1415.67
					D27	26.20	45	2503.99
					D41	14.60	26	1276.51
Erythrocyte band 7 integral membrane protein	**STOM_HUMAN**	22.90	4	215.79	D6	12.50	3	163.46
					D41	22.90	4	172.60
T-complex protein 1 subunit eta	**TCPH_HUMAN**	6.10	3	110.65	D27	6.10	3	110.65

Conclusions

The present studies were conducted to identify the proteins affected by oxidative modifications, in particular the oxidation of the cysteine residues and the introduction of carbonyl functions on amino-acids side chains. The oxidative modification of cysteine thiol group appeared to accumulate in cytosolic proteins along the storage period. However, not all proteins were affected, and affected proteins did not respond equally to this modification. Indeed, most identified proteins in spots presenting IR signal variation (56 %) were specifically identified as suffering from either reversible or irreversible cysteine oxidation. As a consequence, different protein populations were identified regarding this reversibility. Molecular functions engaged by identified proteins are the same but seem differentially represented in these populations. For instance, OR are less abundant in the population related to irreversible oxidation. However, the AO enzyme catalase was specifically identified as target of this oxidative cysteine alteration. As for proteins targeted by reversible cysteine oxidation, antioxidant-related activity presented a higher proportion, as shown by the specifically identified peroxidase-like activities-presenting peroxiredoxin-1 and DJ-1 proteins. Cysteine sulfenylation is the mechanism of action of most antioxidant enzymes thus it is not surprising that constituents of this function family are found more abundant in this population. The OR function is the most relevant example of differentially affected proteins due to the nature of the studied modification, but other functions seem to be targeted as well by reversible (transporters and peptidase regulators) or irreversible (catalytic enzymes other than OR, and phosphatase regulator) protein cysteine oxidation.

The second part of this study dealt with carbonylated proteins in cytosol and membranes. The localization- and time-dependent characters of this modification were here incremented with a protein targeting specificity. If membranes were more affected by quantitative changes of carbonylated content, the soluble fraction of stored RBCs appeared more prone to qualitative changes. In particular, several OR enzymes appeared carbonylated only from the beginning of the second half of the storage period. In membranes, on the contrary, the carbonylation seemed to equally affect the protein population throughout the storage period. One can

however note a higher abundance of carbonylated members of transport proteins and bound cytoskeletal constituents at day 6. This is not in contradiction with the membrane-related quantitative changes revealed in Part 2 because these results are a matter of qualitative changes. More carbonylation can very well occur without changing the proportion of affected molecular functions, *i.e.* more copies of same proteins become carbonylated, or one can easily observe several carbonylation sites on a unique protein due to the high spectrum of potential amino acid targets.

Protein oxidative modification thus seems not to be a global massive phenomenon but is most likely to target specific proteins or protein functions, in addition to a time- and compartment-dependent evolution.

References

1. Mirzaei, H. and F. Regnier, *Affinity chromatographic selection of carbonylated proteins followed by identification of oxidation sites using tandem mass spectrometry.* Anal Chem, 2005. **77**(8): p. 2386-92.
2. Mirzaei, H. and F. Regnier, *Identification of yeast oxidized proteins: chromatographic top-down approach for identification of carbonylated, fragmented and cross-linked proteins in yeast.* J Chromatogr A, 2007. **1141**(1): p. 22-31.
3. Yoo, B.S. and F.E. Regnier, *Proteomic analysis of carbonylated proteins in two-dimensional gel electrophoresis using avidin-fluorescein affinity staining.* Electrophoresis, 2004. **25**(9): p. 1334-41.
4. Mirzaei, H., et al., *Identification of oxidized proteins in rat plasma using avidin chromatography and tandem mass spectrometry.* Proteomics, 2008. **8**(7): p. 1516-27.
5. Mirzaei, H. and F. Regnier, *Enrichment of carbonylated peptides using Girard P reagent and strong cation exchange chromatography.* Anal Chem, 2006. **78**(3): p. 770-8.
6. England, K., C. O'Driscoll, and T.G. Cotter, *Carbonylation of glycolytic proteins is a key response to drug-induced oxidative stress and apoptosis.* Cell Death Differ, 2004. **11**(3): p. 252-60.
7. Lee, S., et al., *Method to site-specifically identify and quantitate carbonyl end products of protein oxidation using oxidation-dependent element coded affinity tags (O-ECAT) and nanoliquid chromatography Fourier transform mass spectrometry.* J Proteome Res, 2006. **5**(3): p. 539-47.
8. Madian, A.G. and F.E. Regnier, *Proteomic identification of carbonylated proteins and their oxidation sites.* J Proteome Res, 2010. **9**(8): p. 3766-80.
9. Oliver, C.N., et al., *Age-related changes in oxidized proteins.* J Biol Chem, 1987. **262**(12): p. 5488-91.
10. Agarwal, S. and R.S. Sohal, *Relationship between susceptibility to protein oxidation, aging, and maximum life span potential of different species.* Exp Gerontol, 1996. **31**(3): p. 365-72.
11. Chao, C.C., Y.S. Ma, and E.R. Stadtman, *Modification of protein surface hydrophobicity and methionine oxidation by oxidative systems.* Proc Natl Acad Sci U S A, 1997. **94**(7): p. 2969-74.
12. Saurin, A.T., et al., *Widespread sulfenic acid formation in tissues in response to hydrogen peroxide.* Proc Natl Acad Sci U S A, 2004. **101**(52): p. 17982-7.
13. Charles, R.L., et al., *Protein sulfenation as a redox sensor: proteomics studies using a novel biotinylated dimedone analogue.* Mol Cell Proteomics, 2007. **6**(9): p. 1473-84.
14. Leonard, S.E., K.G. Reddie, and K.S. Carroll, *Mining the thiol proteome for sulfenic acid modifications reveals new targets for oxidation in cells.* ACS Chem Biol, 2009. **4**(9): p. 783-99.
15. Yang, H.Y., et al., *In-depth analysis of cysteine oxidation by the RBC proteome: advantage of peroxiredoxin II knockout mice.* Proteomics, 2012. **12**(1): p. 101-12.
16. Conesa, A., et al., *Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research.* Bioinformatics, 2005. **21**(18): p. 3674-6.
17. Deisseroth, A. and A.L. Dounce, *Catalase: Physical and chemical properties, mechanism of catalysis, and physiological role.* Physiol Rev, 1970. **50**(3): p. 319-75.

18. Maines, M.D. and G.M. Trakshel, *Purification and Characterization of Human Biliverdin Reductase.* Archives of Biochemistry and Biophysics, 1993. **300**(1): p. 320-326.
19. Chang, S., B. Duerr, and G. Serif, *An epimerase-reductase in L-fucose synthesis.* Journal of Biological Chemistry, 1988. **263**(4): p. 1693-1697.
20. Chae, H.Z., S.J. Chung, and S.G. Rhee, *Thioredoxin-dependent peroxide reductase from yeast.* Journal of Biological Chemistry, 1994. **269**(44): p. 27670-27678.
21. Taira, T., et al., *DJ-1 has a role in antioxidative stress to prevent cell death.* EMBO Rep, 2004. **5**(2): p. 213-8.
22. Chen, J., L. Li, and L.S. Chin, *Parkinson disease protein DJ-1 converts from a zymogen to a protease by carboxyl-terminal cleavage.* Hum Mol Genet, 2010. **19**(12): p. 2395-408.
23. Wilson, M.A., *The role of cysteine oxidation in DJ-1 function and dysfunction.* Antioxid Redox Signal, 2011. **15**(1): p. 111-22.
24. Bosman, G.J., et al., *The proteome of red cell membranes and vesicles during storage in blood bank conditions.* Transfusion, 2008. **48**(5): p. 827-35.
25. Lutz, H.U., S.C. Liu, and J. Palek, *Release of spectrin-free vesicles from human erythrocytes during ATP depletion. I. Characterization of spectrin-free vesicles.* J Cell Biol, 1977. **73**(3): p. 548-60.
26. Borghi, N. and F. Brochard-Wyart, *Tether extrusion from red blood cells: integral proteins unbinding from cytoskeleton.* Biophys J, 2007. **93**(4): p. 1369-79.
27. Kriebardis, A.G., et al., *Progressive oxidation of cytoskeletal proteins and accumulation of denatured hemoglobin in stored red cells.* J Cell Mol Med, 2007. **11**(1): p. 148-55.
28. Wagner, G.M., et al., *Spectrin oxidation correlates with membrane vesiculation in stored RBCs.* Blood, 1987. **69**(6): p. 1777-81.
29. Rank, B.H., N.L. Moyer, and R.P. Hebbel, *Vesiculation of sickle erythrocytes during thermal stress.* Blood, 1988. **72**(3): p. 1060-3.
30. Kriebardis, A.G., et al., *RBC-derived vesicles during storage: ultrastructure, protein composition, oxidation, and signaling components.* Transfusion, 2008. **48**(9): p. 1943-53.

PART 4.

ASSESSMENT OF RBCs DEFENSE MECHANISMS AGAINST OXIDATIVELY MODIFIED PROTEINS

Table of contents

List of illustrations

Introduction

All aerobic living systems are challenged by the production of ROS, causing oxidative injuries to nucleic acids, lipids and proteins [1]. Cells, and in particular RBCs, are well equipped to deal with these damaging species, relying on enzymatic (*e.g.* superoxide dismutase, catalase, glutathione peroxidase) as well as non-enzymatic defenses (*e.g.* glutathione, vitamin C). For a review on cellular defenses against ROS, see reference [2]. In addition to ROS removal or reduction to less reactive species, cells also possess mechanisms to repair (*e.g.* regarding protein oxidation, methionine sulfoxide reductase, thioredoxin, sulfiredoxin, and refolding chaperones) or eliminate (*e.g.* proteasome or vacuolar proteolysis, microvesicle release) oxidized contents [3, 4].

In general, a limited chronic challenge by ROS induces the deployment of the above-mentioned defense mechanisms. In case of prolonged stress condition, ROS production can overwhelm all these defenses, leading to a situation of oxidative stress. At the protein level, the accumulation of oxidative damages involves structural changes, inducing loss of functions and tendency to aggregation. Aggregates of misfolded proteins are involved in and are thought to be responsible for the clinics of several age-related degenerative pathological conditions such as Alzheimer's or Parkinson's disease [5, 6]. It thus seems clear that protein aggregates are deleterious for the cells, and cell incapacity to prevent or eliminate such protein structures can lead to critical situations, as detailed for amyloid fibrils [7]. However, the formation of protein aggregates is counterbalanced by cell defense mechanisms. If the "primary" defense composed of ROS scavengers and oxidoreductant enzymes are not able anymore to prevent the accumulation of oxidized proteins, these modified proteins can be degraded by proteolysis [8] or eliminated by microvesiculation [4].

The proteasome is the catalytic effector of the ubiquitin-proteasome pathway, responsible for the highly controlled proteolysis of misfolded or denatured proteins, playing a major role in numerous cell processes such as cell cycle regulation, apoptosis or immune response [9]. Targeted proteins are specifically addressed to the proteasome through a polyubiquitination

process involving three enzymes responsible for activation, conjugation and binding of the ubiquitin monomers on the protein to be degraded [10, 11]. Ubiquitination allows survival of other proteins, ensuring the selectivity and time-dependent degradation of target proteins [12]. Additionally, the 20S catalytic core (P20S), a barrel-shaped structure of four stacked rings of seven subunits each, has an opening diameter of 13 Å, and an internal catalytic chamber of 53 Å of diameter [13, 14]. As a consequence, native proteins cannot freely enter the P20S, unless being first recognized thanks to the polyubiquitin signal, then ATP-dependently denatured by subunits of the 19S regulator.

In the case of oxidative stress, this “contract murderer” system can lose its targeting-based procedure. Functional P20S was evidenced in mature RBCs [15], and a degradation pathway independent of ATP and ubiquitin was highlighted in these cells [16, 17]. In addition, the protein oxidative denaturation-induced exposure of hydrophobic moieties [18, 19] was shown to be the signal for such degradation [20]. Throughout the storage period of RBCs, a slight loss in intracellular ATP was observed by Hess [21] and Högman [22]. These trends were observed in AS-1 and Erythro-Sol additive solutions, respectively. However, storage in AS-3 let Bennett-Guerrero *et al.* quantifying an ATP loss of 55 % [23]. When stored in SAGM, preliminary results of an LC-MS-based metabolomic study of ECs aging conducted at the SRTS-VD tend to show higher progressive ATP-depletion, of 35 %, 85 % and 95 % at days 6, 27 and 41 of the storage period, respectively (unpublished data). ATP-depletion conditions were shown to induce the dissociation of proteasome complexes (P20S catalytic core dissociates from its main regulator, 19S) [24]. As a consequence, along with the RBC storage period, the proteasome complexes should tend to a higher proportion of P20S catalytic cores presenting a ubiquitin- and ATP-independent proteolytic activity.

Another cellular mechanism, whose defensive role is suggested but still requires clarification, is the microvesicle release [25]. Microvesicles are characterized by their rich membrane phospholipid content, jailing part of the cytoplasmic material from their mother cells [26], and a size ranging from 100 nm to 1 µm. Originating from RBCs, leukocytes, platelets, or endothelium cells, MVs are naturally present in the bloodstream of healthy people, and the variation of their

concentration was characterized, though not specifically, in some pathologies [27-29]. Described long as cell dusts, several biological functions were finally attributed to these MVs, more particularly in thrombosis, hemostasis, or inflammation [30-32]. Similarly to the ferryman Charon carrying souls of the newly deceased across the hell river Acheron towards the land of the dead, a concept of transporter has emerged, in which MVs would be the rowing boat navigating through the bloodstream to introduce lost proteins to the final judgment of liver Kupffer cells [33]. In addition, MVs seem allowing intercellular signal exchange through their externalized phospholipids and/or proteins, or their intern content [34-36]. The direct release in the bloodstream allows fast signal delivery to target cells or organs, even far away from the emitting cell, as hormones do. Physiology of blood MVs has been recently reviewed by Tissot and colleagues, see reference [29].

Regarding the blood banking of RBCs, MV release was characterized as an irreversible rheological storage lesion, presenting a storage-related 20-fold increase at the end of the storage period [37]. Proteomic studies allowed hypothesizing the microvesiculation as a protective mechanism, since altered band 3, the molecular origin of immunological clearance of aged RBCs *in vivo* [33, 38, 39], was characterized in abundance in MVs [25, 26]. The release of MVs thus allows erythrocytes to get rid of removal signals and to postpone their clearance by liver Kupffer cells [25].

This part of the present PhD thesis aims to assess the RBCs capacity to handle intracellular oxidized protein content. In particular, below-presented experiments will focus on the RBCs 20S proteasomal activity throughout their blood banking, as well as their susceptibility to increasing quantities of oxidized protein content. In a second chapter, the elimination of oxidized proteins through the microvesiculation process will be addressed and its impact on whole EC oxidation will be discussed.

Chapter one

Characterization of ATP- and ubiquitin-independent proteolytic degradation through the multi-catalytic complex 20S proteasome (P20S) in stored RBCs

This chapter refers to the article

"Storage induced inhibition of the stored red blood cells multicatalytic proteinase complex proteasome is due to the accumulation of oxidized proteins"

In preparation

Study design

The RBCs proteolytic capacity was investigated by the assay of chymotrypsin-like activity of the 20S proteasome complex. Soluble extracts collected from three ECs at short, intermediate and long storage durations were tested. In addition, this activity was challenged in young samples by the addition of exogenous oxidized proteins.

Material and methods

Sample extracts

Soluble extracts were obtained from EC1, EC2 and EC3 (see details in annex A), as described in Part 2, Chapter 1. Soluble extracts were used as is, without the Hb-depletion step.

Protein oxidation

Solution of commercial human Hb and BSA were prepared in P20S assay buffer (50 mM Tris/HCl, pH 7.5, 25 mM KCl, 10mM NaCl, 1 mM $MgCl_2$). Proteins were chemically oxidized by incubation with 1 volume of 2 X oxidation buffer (50 mM HEPES, pH 7.2, 200 µM FeCl3, 50 mM ascorbic acid), under agitation during 5 h at 37 °C. Then, aggregates were spun down and supernatant were dialyzed overnight against P20S assay buffer at 4 °C. Protein concentrations were determined by the Bradford protein assay for BSA solutions and by the method of Harboe for Hb solutions (see details in Part 2, Chapter 2).

In order to evaluate their behavior to chemical oxidation, Hb and BSA preparations were assayed, after treatment, for their protein carbonyl content by western blot, as described in Part 2, Chapter 2.

P20S activity assay

Soluble proteins extracts (300 µg in P20S assay buffer) were incubated with 200 µM of the fluorogenic peptide substrate Suc-LLVY-AMC, at 37 °C during 4 h. This substrate is specific for cleavage by the catalytic subunits bearing chymotrypsin (ChT)-like protease activity, hereafter referred to as P20S activity. To specifically assess the P20S activity, experiments were also conducted in the presence of 0.5 µM of epoxomicin, a natural highly specific inhibitor of the P20S [40]. The inhibited conditions allow the fluorescence background substraction, to get rid of signal from buffer, sample, substrate auto-degradation, and substrate unspecific degradation by

other proteases. The substrate cleavage by proteases from the samples allows the release of the 7-amino-4-methylcoumarin (AMC) molecule. Kinetics of AMC release was measured over time by fluorescence detection at 442 nm upon excitation at 342 nm, on a SPECTRAmax® GEMINI XS microplate spectrofluorometer (Molecular Devices Corporation, Sunnyvale, CA, USA). Standards of AMC dilutions allowed determining a conversion factor (in µM/A.F.U.) for P20S activity calculation in samples. Additionally, a standard of dilutions of commercial 20S proteasome, purified from human erythrocytes, allowed estimating the quantity of active P20S in samples.

To determine the effect of exogenous oxidized proteins on P20S activity, day 6 samples were spiked with different quantities of oxidized BSA or oxidized Hb, corresponding to different carbonyl contents. Control conditions where samples were spiked with same amounts of untreated proteins were also conducted.

Statistics

Effect of storage on the P20S activity was assessed by one-way ANOVA followed by a Tukey HSD test for pairwise multiple comparisons between each storage duration couple. Statistics were evaluated using the free open source RStudio IDE software (version 0.97.551).

Results and discussion

The P20S activity assays were realized in absence of ATP which is required for the unfolding and ubiquitin-recycling activities bore by the subunits of the main 19S regulator complex. In ATP-depleted conditions, such as occurring during the storage of RBCs, the proteasomal degradation is thus mainly assumed by the 20S catalytic core on its own, and protein degradation occurs independently of the ubiquitination targeting. Indeed, P26S (20S core particle, CP, associated with 19S regulatory particles, RP) dissociates under ATP-depletion conditions, as revealed by Tanaka and colleagues [24] through the loss of substrate degradation in corresponding glycerol density gradient fractions, in comparison with ATP-regeneration conditions. Moreover, they have shown that immunodetection signal of 20S and 19S subunits shifted from fast sedimenting fractions (26 S) to low sedimenting ones (20 S) upon ATP depletion. The following results are thus assumed to be mainly related to the 20S CP only. This is an important point to consider because the small-sized Suc-LLVY-AMC peptide substrate used here does not need ATP-dependent denaturation to enter the proteasome catalytic core and would be degraded by P26S if such complexes were stable in ATP-depletion conditions.

Determination of P20S activity during RBCs storage

The assay of P20S activity in stored RBCs reveals a late-occurring change during the storage period (see Figure 32). The triplicate measures of P20S activities from three ECs sampled at days 6, 27 and 41 of storage shows similar mean activities determined as $2.42\pm0.8\cdot10^{-3}$ and $2.40\pm0.7\cdot10^{-3}$ $pmol\cdot min^{-1}\cdot\mu g^{-1}$ at days 6 and 27, respectively. A mean activity of $1.44\pm0.5\cdot10^{-3}$ $pmol\cdot min^{-1}\cdot\mu g^{-1}$, measured at day 41, represents a decrease of 40.5 % in comparison with the activity at day 6. This decreasing activity could be explained either by a quantitative loss of proteasome complexes (degradation of the multicatalytic complexes) or by a loss of function due to the inhibition of the enzyme/substrate interaction (protein modification, blocking of the access to the catalytic chamber). During RBCs storage, the ATP depletion tends to prevent the ubiquitin-targeted degradation of misfolded proteins, due to dissociation of the P26S complexes, as mentioned above. Moreover the present study shows an inhibition of the P20S activity at the end of the storage period. This inhibition decreases the less controlled

degradation of mildly oxidized proteins, supporting their accumulation, over-oxidation and aggregation in aging stored RBCs.

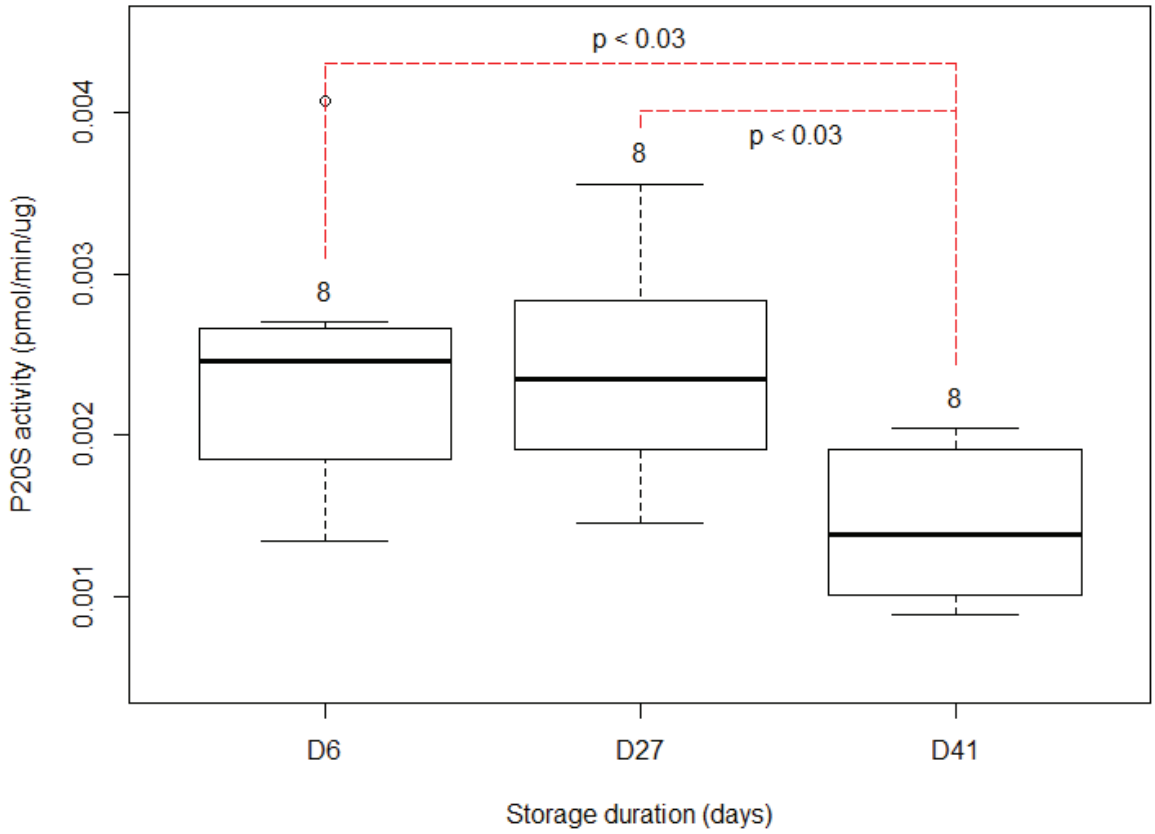


Figure 32: 20S proteasome Chymotrypsin-like activity in stored RBCs. P20S activity was assessed in stored RBCs collected at days 6, 27 and 41 of the storage of three ECs, through kinetics of the ChT-like proteolytic degradation of the specific fluorogenic substrate Suc-LLVY-AMC. The ANOVA analysis revealed a significant effect of the storage duration ($p \cong 0.014$), with a significant difference at day 41, in comparison with day 6 ($p \cong 0.025$) and day 27 ($p \cong 0.029$), as revealed by pairwise multiple comparison test.

Effect of exogenous oxidized proteins on P20S activity in short-stored RBCs

As shown in Part 2, along with the increasing storage duration comes an increasing oxidation of RBC proteins. This accumulation of oxidized material is amplified by the inhibition of their degradation machinery, the 20S proteasome. Protein hydrophobic moieties exposure through oxidative modifications allows their interaction with the P20S. This interaction was shown to

favor the degradation, but without the ATP-dependent denaturation, one can wonder whether oxidized material binding could also trigger the inhibitory effect shown above. Indeed, Pacifici and colleagues have shown that the more hydrophobe damaged-Hb, the more and faster degraded by proteasome [20]. However, they also documented that hemin, a highly hydrophobic molecule characterized by a structure very similar to Hb, presents a strong inhibitory effect on proteasome-mediated degradation of oxidized proteins [20].

Day 6 sample from EC1 was mixed with different quantities of exogenous untreated or oxidized Hb (Figure 33A) or BSA (Figure 33B). The addition of exogenous untreated proteins induces a little decrease in the measured activity of the day 6 sample, compared to the control sample (see Figure 33). The increase in quantity of untreated exogenous proteins does not seem to induce any significant activity variation (three first light histograms, Figure 33). On the contrary, the P20S activity appears to be decreased by rising quantities of oxidized exogenous proteins, as shown in Figure 33 (three first dark histograms). If both oxidized Hb and oxidized BSA induce this dose-dependent activity decrease, the Hb-associated inhibition seems higher than the BSA one. Indeed, the addition of 50 µg of oxidized Hb induces a loss of 18 % of P20S activity in comparison with the same amount of untreated Hb (Figure 33A). As for oxidized BSA, the addition of 150 µg only produces a 10 % loss of activity when compared to the same amount of untreated BSA (Figure 33B). The oxidized versions of the two tested proteins, Hb and BSA, seems thus to have an inhibitory effect on the proteasomal degradation of the Suc-LLVY-AMC peptide substrate, in ATP-depleted conditions. However, it seems that oxidized Hb has a higher inhibitory effect than oxidized BSA. It is possible that these two proteins differentially react to the same chemical oxidation process. The two oxidized protein preparations were thus assessed for protein carbonylation, and were found to be equally carbonylated at around 500 fmol/µg (data not shown). The different inhibitory effects observed through the addition of oxidized Hb or oxidized BSA is thus not due to different oxidation rates of these proteins. On the contrary, the proteasome susceptibility to inhibition by oxidized content seems protein-dependent.

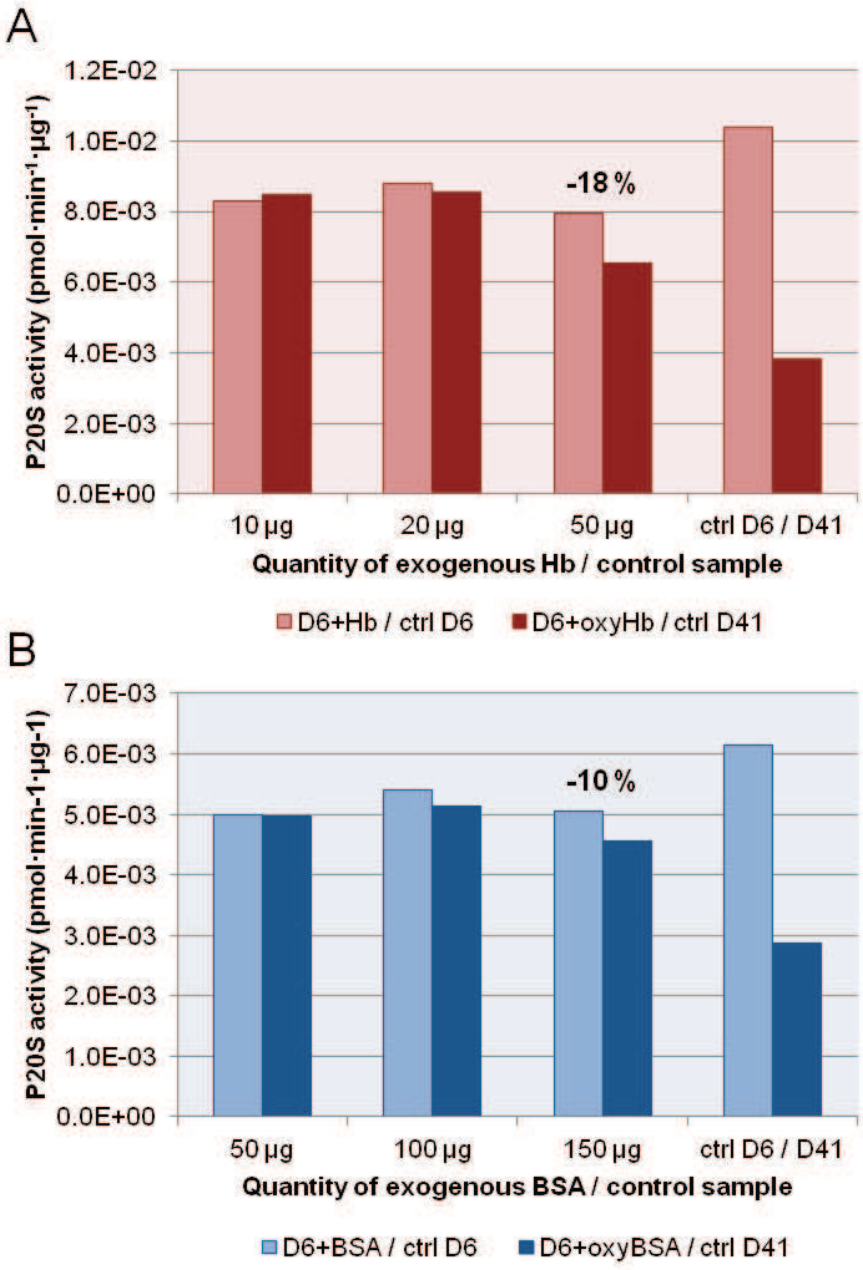


Figure 33: P20S activity in young RBC samples challenged by exogenous oxidized proteins. RBC soluble extracts from short-stored ECs (6 days of storage) were spiked with different quantities of untreated (light histograms) or oxidized (dark histograms) exogenous Hb (A) and BSA (B). Measures were done in triplicate on a unique biological sample.

Chapter two

Elimination of oxidized proteins out of the cell through the microvesiculation process

This chapter refers to the article

"Subcellular fractionation of stored red blood cells reveals a compartment-based protein carbonylation evolution"

Published in

Journal of Proteomics, 76 (2012) 181-193

Study design

MVs were isolated from three short-stored ECs (less than 10 days) as well as from three long-stored ECs (approx. 40-days). Total protein extracts were incubated with 2,4-DNPH for carbonyls derivatization, and modified proteins were quantified by western blot and densitometry. Data were combined with a previous study assessing the quantity of MVs released by RBCs throughout the storage period, in order to estimate the elimination of carbonylated protein content through this mechanism.

Material and methods

Microvesicles enrichment

Three young ECs (EC9, EC10 and EC11, at 5, 8 and 9 days of storage, respectively) and three old ECs (EC12, EC13 and E14, at 38, 39 and 40 days of storage, respectively) were used for this study. Details on blood donors can be found in annex, table 1. ECs were centrifuged at 2000·g during 10 minutes at 4 °C in order to pellet the RBCs. The supernatants were centrifuged once again in the same conditions to eliminate residual erythrocytes. Then, the cell free storage media were ultracentrifuged three times during 1 hour at 120000·g and at 4 °C. Intermediate pellets of MVs were washed in 0.9 % NaCl solution, and the final pellets were resuspended in a small volume (usually 1 mL) of the same solution.

Microvesicles counts

MVs were counted *via* a flow cytometry method developed in our lab [37]. Briefly, MVs were labeled with FITC-coupled mouse anti-human CD47 antibody during 20 minutes under gentle agitation at room temperature. Labeled MVs were then diluted in 0.9 % NaCl into BD Trucount™ Tubes containing a precise number of control micro-beads appearing positive to FITC, PE and PE-Cy5 fluorescence. These triply-labeled beads allow the quantitation of MVs.

Protein extracts and carbonyls quantitation

Total MV proteins were extracted with Laemmli buffer. Protein extracts from $0.5 \cdot 10^6$ MVs were derivatized with 2,4-DNPH, separated by SDS-PAGE and transferred onto PVDF membrane. DNP residues were immunologically detected and carbonylated proteins were revealed by enhanced chemiluminescence with HRP-conjugated secondary antibodies. Films were scanned with a densitometer and intensities of entire migration lanes were determined with the ImageQuant

TL 7.0 software, and compared with a control of known carbonyl amount for sample quantitation. For details, see the material and methods section of Part 2, Chapter 2.

Purification and identification of carbonylated proteins from MV extracts

Total MVs proteins were extracted with DC buffer and Hb was removed by Ni-IMAC. Carbonylated proteins were derivatized with BHZ then purified by avidin-biotin affinity chromatography. Different extracts (total, Hb-depleted, Hb fraction and carbonylated proteins) were separated by SDS-PAGE, and bands of interest were excised, reduced/alkylated and digested for protein identification through LC-MS/MS analyses. Method details can be found in Part 2, Chapter 2 and Part 3, Chapter 2.

Data processing and statistics

Carbonyl detection was expressed as volumes further converted in femtomoles of carbonyls then normalized per MV. To investigate the loss of carbonylated proteins from RBCs, an exponential regression was interpolated between the mean carbonylation values in “young” and “old” MV populations. This hypothetical trend was combined with microvesicle release data published in 2008 by our group [37]. This way, it was possible to estimate the progressive elimination of carbonylated proteins through the microvesiculation process.

Quantitation of protein carbonylation in MVs from young and old ECs was realized in triplicate (both biological and technical). The reproducibility was evaluated and a mean CV % was determined as 7.2 %. The mean difference of carbonyl content between short stored- and long-stored purified MVs was statistically determined using the Student paired *t* test with a 2-tailed distribution. Data was considered statistically significant for *p*-values below 5 %.

Results and discussion

Western blots of carbonylated proteins revealed the presence of such modified proteins inside and/or at the surface of MVs released by RBCs during their blood banking, in young as well as in old ECs. Figure 34 shows the immunodetection pattern of SDS-PAGE-separated carbonylated proteins extracted from $0.5 \cdot 10^6$ MVs collected in three ECs stored for less than 10 days and in three ECs stored for approximatively 40 days. Panel A shows the Ponceau Red staining of total MV proteins efficiently transferred onto the membrane and panel B corresponds to the immunodetection of derivatized, *i.e.* carbonylated, proteins. Interestingly, Figure 34A reveals higher Hb content (globin monomers and dimers at around 15 and 30 kDa, respectively) in MVs isolated from long-stored ECs, in comparison with short-stored ones. This is in adequation with the progressive binding of denatured hemoglobin at the inner face of erythrocyte membranes during RBCs storage [41-43] and with the main membrane-related MVs protein composition [25, 26]. Detecting more Hb in "old" MVs suggest that its loci of membrane binding should, if not playing an active role, at least be included in the cell wastes eliminated by the microvesiculation process.

Contrary to the unspecific and low-sensitivity Ponceau Red protein staining, the immunodetection of carbonylated content in these MV extracts reveals oxidized material throughout the molecular weight distribution, either present as quite resolved bands or as diffused signal, with a global high background-like signal in the high MW, relevant of aggregated material. The overall detection signal of these modified proteins clearly appears more intense in "old" MV extracts than in "young" MV ones, as shown in Figure 34B. The densitometry quantitation of the carbonylated protein content highlights a two-fold increase in favor of the "old" MV extracts, as presented in Figure 35 (0.013 and 0.026 fmol/MV in "young" and "old" MVs, respectively; n=9). Student *t* test reveals this difference as statistically significant with a 1 % risk of error. Carbonylation quantitation on the monomer Hb signal reveals a similar 2-fold ratio between "young" and "old" MVs (data not shown). Thus carbonylated Hb does not seem more oxidized with time, but more eliminated by MVs. This is coherent with the steady state pattern evolution of Hb carbonylation shown in Figure 9 in Part 2, Chapter 2.

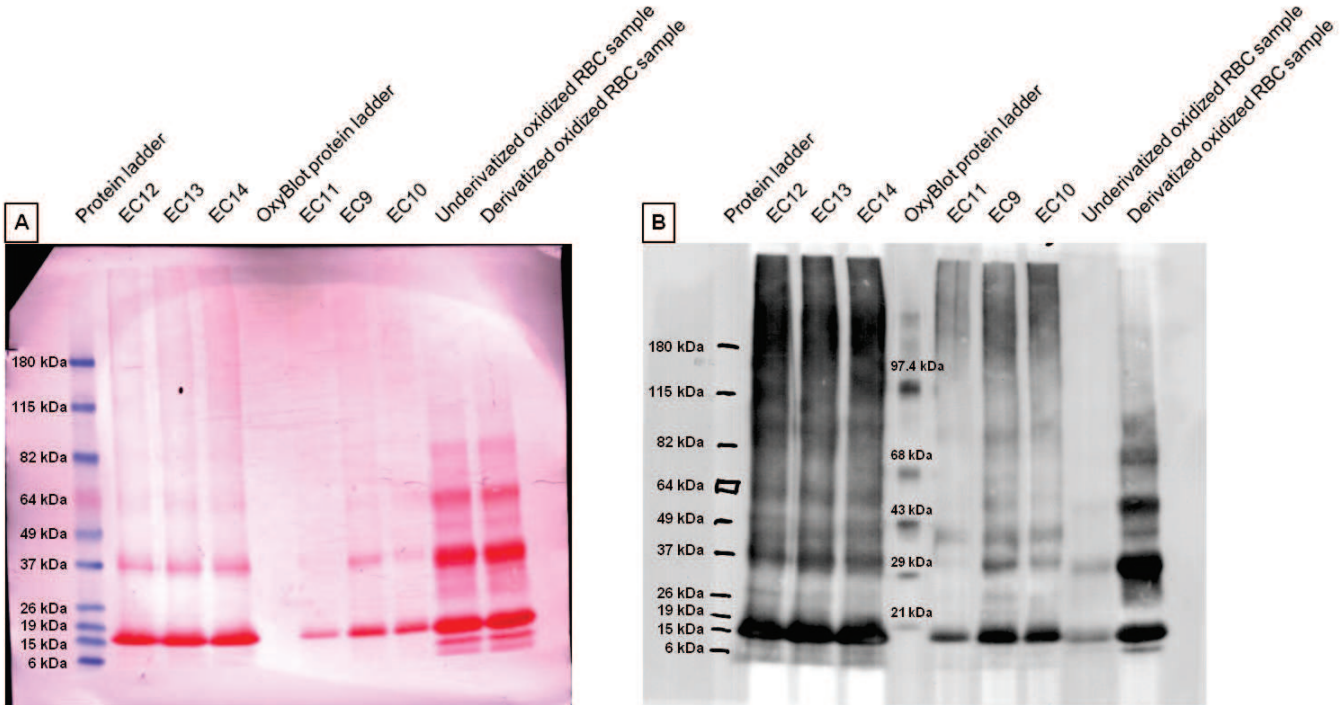


Figure 34: Western Blot detection of carbonylated proteins in MVs. MVs were isolated from short- (EC9, 10 and 11) or long- (EC12, 13 and 14) stored ECs. Carbonylated proteins were derivatized with 2,4-DNPH and whole extracts were separated by SDS-PAGE, as well as chemically oxidized RBC sample for positive (derivatized) and negative (underivatized) controls. A protein ladder and a mixture of carbonylated proteins were included for molecular weight scaling and quantification of samples protein carbonylation, respectively. Panel A shows the Ponceau Red staining of total proteins and panel B shows the immunodetection of derivatized carbonylated proteins.

The depletion of Hb from MV proteins extracted with DC reveals residual Hb monomers as well as low MW material below 10 kDa (see Figure 36). The avidin-biotin capture of derivatized proteins from Hb-depleted MV extract allows the elution of these fragments and residual Hb as carbonylated material, as shown in Figure 36. The presence of relatively high amount of residual hemoglobin is explained by the altered capacity of oxidized Hb to be retained by the Nickel-loaded IMAC column (see annex C).

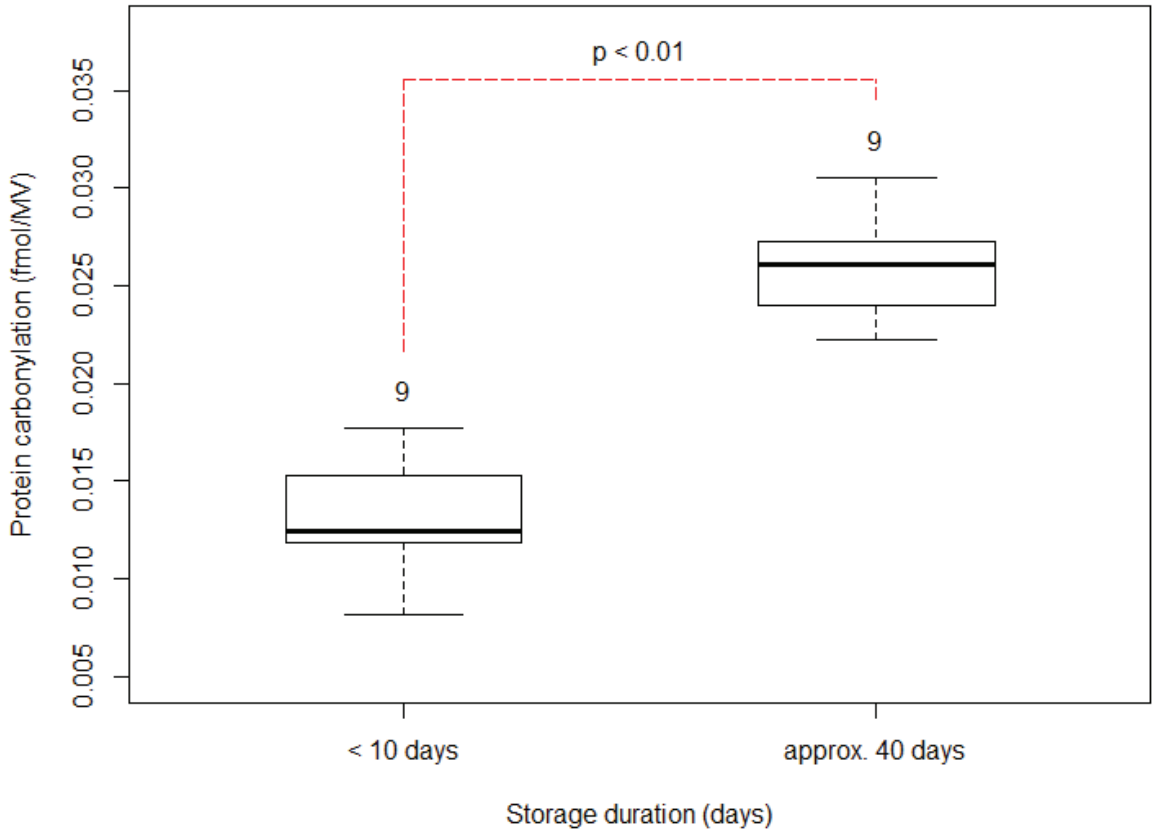


Figure 35: Quantitation of protein carbonylation in MVs collected from short- and long-stored RBCs. The boxplots represent the mean quantitation of protein carbonylation, expressed in fmol/MV, in ECs stored for less than 10 days and ECs stored for approximatively 40 days. Measures were done in triplicate and on three biological replicates. The mean fold change of 1.89 in favor of MVs from long-stored ECs was shown statistically significant with a 1 % risk factor (Student paired t test).

MVs reveal carbonylated protein content which level evolves when comparing MVs isolated from ECs of different storage durations (Figure 34 and Figure 35). Carbonylated content inside MVs thus seems to be affected by the duration of storage of RBCs, as intracellular RBCs compartments were shown to be in Part 2. Microvesiculation process is thought to be a protective mechanism for the aging RBCs [25]. It is difficult to say whether carbonylated proteins are actively eliminated through this process, or if they are just passively embedded because of spatial proximity. Nevertheless, higher protein carbonylation rates inside MVs compared to intracellular RBC compartments, even in short-stored ECs, seem to imply a driven elimination. The membrane recruitment, such as in the case of hemichromes (aggregated oxidized Hb), could play this “targeting” role for elimination.

Determination of protein concentration in MVs total extracts shows a mean protein content of 9 pg/MV, whatever the storage duration of the sampled EC (data not shown). The mean protein carbonylation rates in “young” and “old” MVs are thus determined of 1.44 and 2.78 pmol/µg, respectively (Figure 35). These values are of the same order of magnitude but higher than the rates determined for RBCs membrane fractions (0.3 to 0.5 pmol/µg and 0.5 to 1 pmol/µg for integral membrane and cytoskeleton, respectively, as shown in Figure 9 in Part 2, Chapter 2), indicating a probable specific elimination mechanism.

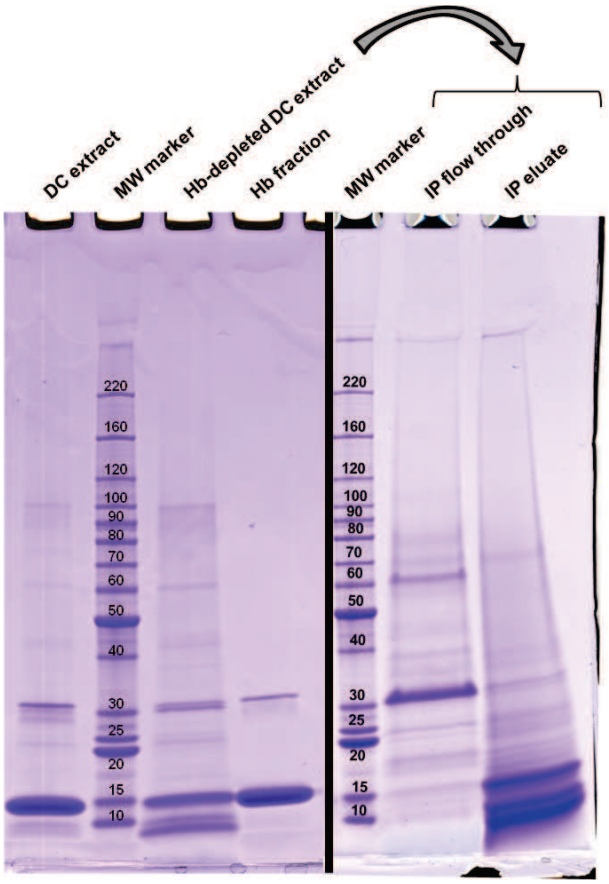


Figure 36: 1D-PAGE patterns of hemoglobin depletion and capture of carbonylated proteins from MV extract. MV proteins were extracted with DC buffer (lane 1), and Hb was depleted by nickel-based IMAC (lanes 3 and 4). Proteins from Hb-depleted extract were derivatized with biotin-hydrazide to purify carbonylated proteins (lanes 6 and 7).

If it is relatively easy to quantify MVs from short-stored ECs (a few µL are needed), their low absolute count requires the sacrifice of “young” ECs to collect enough material for further analyses. It is thus hardly feasible to undertake follows-up of MVs throughout the storage of

given ECs. With that consideration in mind, an estimation of the carbonylation loss throughout the storage period was carried out, based on the protein carbonyls data and MVs counts data obtained in our lab in 2008 [37]. Data of protein carbonyls in "young" and "old" MVs were extrapolated with an exponential regression model. The exponential model $y = a * e^{b*x}$ can be expressed as $y = a * (e^x)^b$. The low b coefficient ($b \cong 0.0198$) makes the model nearly linear for $x \in]0; 42]$ (ECs storage duration). The extrapolation with a linear regression model (data not shown) gave similar results to what is presented below. The exponential model estimating the quantity of carbonylated proteins in MVs throughout the storage period is shown in Figure 37A (blue circles), as well as the counts of MVs per EC (red spots). The latter clearly shows the exponential pattern of the release of MVs, particularly increasing after 4 weeks of storage. Combining these data, an estimation of protein carbonyls lost per EC through the microvesiculation process is shown in Figure 37B. A similar 4 weeks delayed exponential trend is observed. Of interest is the carbonylation loss in the end of the storage period, *i.e.* at 42 days, which is comprised between 200 and 300 nmol/EC. This amount of carbonyl content loss is coherent with the decrease observed in integral membrane fraction in the end of the RBCs storage (Part 2, Chapter 2).

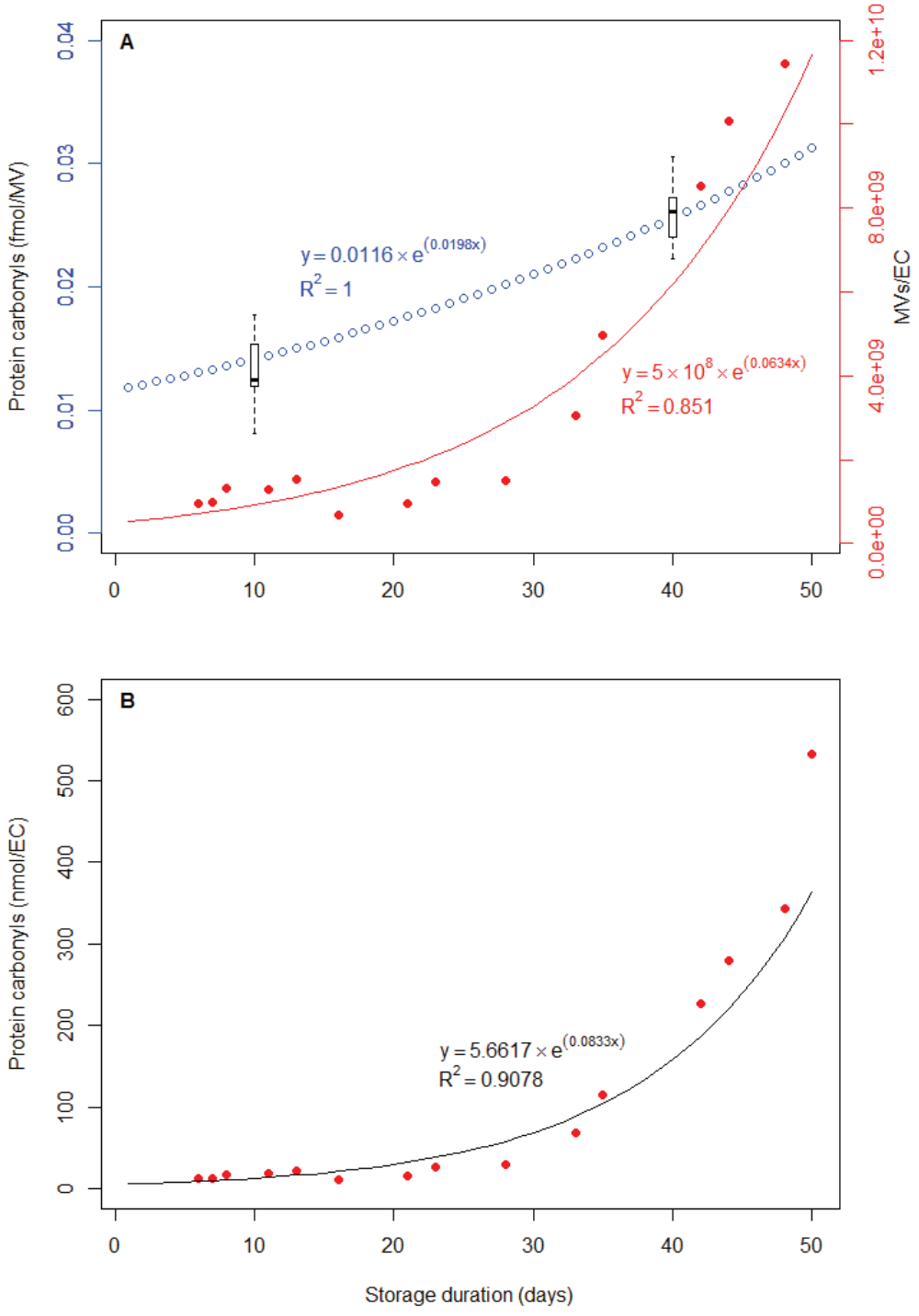


Figure 37: Simulation of protein carbonylation loss by microvesiculation. Panel A: blue circles represent the exponential regression of carbonylated protein content in microvesicles along the storage (left axis), with in black boxplots the experimental values. Red spots are adapted data from a previous study investigating the quantity of MVs in ECs along the storage (right axis), with the corresponding exponential regression (red curve). Combined together in panel B, these data give an estimation of the loss of carbonylated proteins from RBCs by the MVs (calculated values in red spots and theoretical exponential trend in black curve).

Conclusions

Handling oxidized protein content is pivotal for cells, since accumulation and aggregation of such modified material can lead to severe pathologies. The main machinery responsible for degradation of misfolded or denatured proteins involves the multicatalytic complex proteasome. In the transfusion context, proteomic studies reported EC storage-related changes in proteasome content [26, 44], suggesting a potential alteration of its proteolytic capacities.

Through the present study, we were able to highlight a P20S-driven proteolysis activity, out of the ATP-dependent ubiquitin-proteasome pathway. In RBCs extracts, ATP levels of the low millimolar range were shown to allow for the dissociation of up to 70 % of existing P26S complexes [45]. Such complexes should not be attributed to the ATP- and ubiquitin-independent degradation of oxidized proteins. ATP depletion conditions were thus applied to measure only proteasomal chymotrypsin-like activity from the dissociated 20S core particles. To the best of our knowledge, only one study, conducted in 2008 by Majetschak and Sorell, questioned the proteasomal activity in stored RBCs, as a biological application of methods developed for quantitation and characterization of proteasome complexes [45]. They observed a stable content and a constant activity of 20S complexes over the whole storage period, and a recruitment of 26S complexes during the first 3 weeks of storage period to attain a plateau, though not relating activity modification. However, the specific P26S activity (activity normalized by concentration) revealed a rapid inhibition during storage. This study was conducted on CPD-anticoagulated AS-1-stored RBCs. The AS-1 additive solution mainly differs from the SAGM one by higher adenine and lower glucose contents (2 *vs* 1.25 mM and 45 *vs* 111 mM, respectively). As a consequence, they only observed a very slight decrease in the intracellular ATP content along the storage period. Both AS-1 and SAGM additive solutions are known to induce different storage behavior of aging stored RBCs [46], probably related to composition-dependent difference of metabolism. It is thus not surprisingly that our results on SAGM-stored RBCs show a different proteasome activity trend over the storage period, with a significant decrease of P20S activity after 4 weeks of storage.

In the oxidizing context of long-stored RBCs, we investigated the impact of increasing quantities of oxidized proteins on the control P20S activity in RBCs stored for 1 week. Preliminary results confirm the hypothesis of the inhibition of proteasomal activity by accumulation of oxidized protein content, recognized by P20S complexes, and likely to spatially hinder the substrate entry to proteasome. A dose-dependent inhibition of P20S activity is thus measured through the addition of increasing content of exogenous oxidized proteins to cytosolic extracts of young RBCs.

Additionally to oxidative events, the inhibition of proteasome activity could find an explanation in assembly capacity issues. In a recent paper, Bousquet-Dubouch *et al.* highlighted a high part of free 20S proteasomes (64 %, mean of nine cell lines), potentially acting as "*a reservoir to which regulators can associate to quickly modulate the proteolysis state in the cell*" [47]. The oxidative stress occurring in stored RBCs may activate such a response, which would be concordant with the P26S recruitment observed by Majetschak during the first 3 weeks of ECs storage, in conditions that seem to better preserve ATP levels [45]. Less free P20S can explain the decreased activity that we highlighted after 4 weeks of storage. Moreover, Bousquet-Dubouch *et al.* also showed that a part of P20S complexes (from 2.2 to 16.5 % depending on the cell line) were in the assembly process, through the co-immunopurification of proteasome assembly chaperones (PAC1 to PAC4 and POMP) [47]. Such chaperones can be targeted by oxidative lesions (as other chaperones were shown to be in Part 3), potentially preventing the assembly of P20S complexes in long-stored RBCs. Investigations in this sense should be performed in banked RBCs.

Shrunk capacities of proteolytic degradation of oxidized protein content can be counterbalanced by physical removal through the microvesiculation process. Indeed, this phenomenon, exponentially increasing throughout the storage period, appears to clear RBCs, at least partially, of their oxidized protein content, with an increasing trend as the storage lasts. MVs from long-stored ECs reveal a carbonylated protein content twice as in MVs from short-stored ECs. The combination of MV counts and quantity of oxidized proteins found in MVs throughout the storage period allows estimating the loss of oxidized proteins through this

process, and to associate it with the evolution of membrane protein carbonyls shown in Part 2, Chapter 2.

Once in an oxidative stress environment, such as a prolonged storage duration seems to be (see supplementary data of ref [48]), RBCs possess mechanism to fight against the accumulation of oxidized material. It seems that proteasome plays a first proteolytic degradation role, while a moderate level of microvesiculation allows elimination of a few amount of oxidized proteins. However, the proteasome machinery seems to be altered, due to a too high level of oxidatively damaged material. Around 4 weeks of storage, concomitant to the decreasing proteolytic capacities, the MV release enters its exponential phase and could continue the garbage work to prevent over-accumulation of oxidized proteins.

References

1. Bandyopadhyay, U., D. Das, and R.K. Banerjee, *Reactive oxygen species: Oxidative damage and pathogenesis.* Current Science, 1999. **77**(5): p. 658-666.
2. Yu, B.P., *Cellular defenses against damage from reactive oxygen species.* Physiol Rev, 1994. **74**(1): p. 139-62.
3. Pacifici, R.E. and K.J. Davies, *Protein, lipid and DNA repair systems in oxidative stress: the free-radical theory of aging revisited.* Gerontology, 1991. **37**(1-3): p. 166-80.
4. Kriebardis, A.G., et al., *RBC-derived vesicles during storage: ultrastructure, protein composition, oxidation, and signaling components.* Transfusion, 2008. **48**(9): p. 1943-53.
5. Chiti, F. and C.M. Dobson, *Protein misfolding, functional amyloid, and human disease.* Annu Rev Biochem, 2006. **75**: p. 333-66.
6. Lansbury, P.T. and H.A. Lashuel, *A century-old debate on protein aggregation and neurodegeneration enters the clinic.* Nature, 2006. **443**(7113): p. 774-9.
7. Stefani, M. and C.M. Dobson, *Protein aggregation and aggregate toxicity: new insights into protein folding, misfolding diseases and biological evolution.* J Mol Med (Berl), 2003. **81**(11): p. 678-99.
8. Grune, T., T. Reinheckel, and K.J. Davies, *Degradation of oxidized proteins in mammalian cells.* FASEB J, 1997. **11**(7): p. 526-34.
9. Ciechanover, A., A. Orian, and A.L. Schwartz, *Ubiquitin-mediated proteolysis: biological regulation via destruction.* Bioessays, 2000. **22**(5): p. 442-51.
10. Hershko, A. and A. Ciechanover, *The ubiquitin system.* Annu Rev Biochem, 1998. **67**: p. 425-79.
11. Nandi, D., et al., *The ubiquitin-proteasome system.* J Biosci, 2006. **31**(1): p. 137-55.
12. Voges, D., P. Zwickl, and W. Baumeister, *The 26S proteasome: a molecular machine designed for controlled proteolysis.* Annu Rev Biochem, 1999. **68**: p. 1015-68.
13. Religa, T.L., R. Sprangers, and L.E. Kay, *Dynamic regulation of archaeal proteasome gate opening as studied by TROSY NMR.* Science, 2010. **328**(5974): p. 98-102.
14. Jung, T. and T. Grune, *The proteasome and the degradation of oxidized proteins: Part I-structure of proteasomes.* Redox Biol, 2013. **1**(1): p. 178-82.
15. Neelam, S., et al., *Functional 20S proteasomes in mature human red blood cells.* Exp Biol Med (Maywood), 2011. **236**(5): p. 580-91.
16. Fagan, J.M. and L. Waxman, *The ATP-independent pathway in red blood cells that degrades oxidant-damaged hemoglobin.* J Biol Chem, 1992. **267**(32): p. 23015-22.
17. Fagan, J.M., L. Waxman, and A.L. Goldberg, *Red blood cells contain a pathway for the degradation of oxidant-damaged hemoglobin that does not require ATP or ubiquitin.* J Biol Chem, 1986. **261**(13): p. 5705-13.
18. Chao, C.C., Y.S. Ma, and E.R. Stadtman, *Modification of protein surface hydrophobicity and methionine oxidation by oxidative systems.* Proc Natl Acad Sci U S A, 1997. **94**(7): p. 2969-74.
19. Davies, K.J. and M.E. Delsignore, *Protein damage and degradation by oxygen radicals. III. Modification of secondary and tertiary structure.* J Biol Chem, 1987. **262**(20): p. 9908-13.

20. Pacifici, R.E., Y. Kono, and K.J. Davies, *Hydrophobicity as the signal for selective degradation of hydroxyl radical-modified hemoglobin by the multicatalytic proteinase complex, proteasome.* J Biol Chem, 1993. **268**(21): p. 15405-11.
21. Hess, J.R., et al., *The effects of phosphate, pH, and AS volume on RBCs stored in saline-adenine-glucose-mannitol solutions.* Transfusion, 2000. **40**(8): p. 1000-6.
22. Hogman, C.F., et al., *Improved maintenance of 2,3 DPG and ATP in RBCs stored in a modified additive solution.* Transfusion, 2002. **42**(7): p. 824-9.
23. Bennett-Guerrero, E., et al., *Evolution of adverse changes in stored RBCs.* Proc Natl Acad Sci U S A, 2007. **104**(43): p. 17063-8.
24. Tanahashi, N., et al., *Hybrid proteasomes. Induction by interferon-gamma and contribution to ATP-dependent proteolysis.* J Biol Chem, 2000. **275**(19): p. 14336-45.
25. Willekens, F.L., et al., *Erythrocyte vesiculation: a self-protective mechanism?* Br J Haematol, 2008. **141**(4): p. 549-56.
26. Bosman, G.J., et al., *The proteome of red cell membranes and vesicles during storage in blood bank conditions.* Transfusion, 2008. **48**(5): p. 827-35.
27. Piccin, A., W.G. Murphy, and O.P. Smith, *Circulating microparticles: pathophysiology and clinical implications.* Blood Rev, 2007. **21**(3): p. 157-71.
28. Simak, J. and M.P. Gelderman, *Cell membrane microparticles in blood and blood products: potentially pathogenic agents and diagnostic markers.* Transfus Med Rev, 2006. **20**(1): p. 1-26.
29. Tissot, J.-D., et al., *Blood microvesicles: From proteomics to physiology.* Translational Proteomics, 2013. **1**(1): p. 38-52.
30. Diamant, M., et al., *Cellular microparticles: new players in the field of vascular disease?* Eur J Clin Invest, 2004. **34**(6): p. 392-401.
31. Rubin, O., et al., *Red blood cell-derived microparticles isolated from blood units initiate and propagate thrombin generation.* Transfusion, 2013. **53**(8): p. 1744-54.
32. Sadallah, S., C. Eken, and J.A. Schifferli, *Ectosomes as modulators of inflammation and immunity.* Clin Exp Immunol, 2011. **163**(1): p. 26-32.
33. Willekens, F.L., et al., *Liver Kupffer cells rapidly remove red blood cell-derived vesicles from the circulation by scavenger receptors.* Blood, 2005. **105**(5): p. 2141-5.
34. Mathivanan, S., H. Ji, and R.J. Simpson, *Exosomes: extracellular organelles important in intercellular communication.* J Proteomics, 2010. **73**(10): p. 1907-20.
35. Mause, S.F. and C. Weber, *Microparticles: protagonists of a novel communication network for intercellular information exchange.* Circ Res, 2010. **107**(9): p. 1047-57.
36. Schifferli, J.A., *Microvesicles are messengers.* Semin Immunopathol, 2011. **33**(5): p. 393-4.
37. Rubin, O., et al., *Microparticles in stored red blood cells: an approach using flow cytometry and proteomic tools.* Vox Sang, 2008. **95**(4): p. 288-97.
38. Hornig, R. and H.U. Lutz, *Band 3 protein clustering on human erythrocytes promotes binding of naturally occurring anti-band 3 and anti-spectrin antibodies.* Exp Gerontol, 2000. **35**(8): p. 1025-44.
39. Kriebardis, A.G., et al., *Storage-dependent remodeling of the red blood cell membrane is associated with increased immunoglobulin G binding, lipid raft rearrangement, and caspase activation.* Transfusion, 2007. **47**(7): p. 1212-20.

40. Groll, M., et al., *Crystal Structure of Epoxomicin:20S Proteasome Reveals a Molecular Basis for Selectivity of α',β'-Epoxyketone Proteasome Inhibitors.* Journal of the American Chemical Society, 2000. **122**(6): p. 1237-1238.
41. Low, P.S., et al., *Characterization of the reversible conformational equilibrium of the cytoplasmic domain of erythrocyte membrane band 3.* J Biol Chem, 1984. **259**(21): p. 13070-6.
42. Waugh, S.M. and P.S. Low, *Hemichrome binding to band 3: nucleation of Heinz bodies on the erythrocyte membrane.* Biochemistry, 1985. **24**(1): p. 34-9.
43. Waugh, S.M., J.A. Walder, and P.S. Low, *Partial characterization of the copolymerization reaction of erythrocyte membrane band 3 with hemichromes.* Biochemistry, 1987. **26**(6): p. 1777-83.
44. Antonelou, M.H., et al., *Red blood cell aging markers during storage in citrate-phosphate-dextrose-saline-adenine-glucose-mannitol.* Transfusion, 2010. **50**(2): p. 376-89.
45. Majetschak, M. and L.T. Sorell, *Immunological methods to quantify and characterize proteasome complexes: development and application.* J Immunol Methods, 2008. **334**(1-2): p. 91-103.
46. Sparrow, R.L., et al., *In vitro measures of membrane changes reveal differences between red blood cells stored in saline-adenine-glucose-mannitol and AS-1 additive solutions: a paired study.* Transfusion, 2014. **54**(3): p. 560-8.
47. Fabre, B., et al., *Label-free quantitative proteomics reveals the dynamics of proteasome complexes composition and stoichiometry in a wide range of human cell lines.* J Proteome Res, 2014. **13**(6): p. 3027-37.
48. D'Alessandro, A., et al., *Time-course investigation of SAGM-stored leukocyte-filtered red bood cell concentrates: from metabolism to proteomics.* Haematologica, 2012. **97**(1): p. 107-15.

PART 5.

CONCLUSIONS AND PERSPECTIVES

Table of contents

List of illustrations

Context of this PhD thesis

Cold-storage of RBCs in additive solution is of high importance for the transfusion medicine field, because it allows the time and space separation of the blood donation and blood transfusion processes. In addition, the conservation of blood products enabled the establishment of blood banks nationwide, centralizing blood resources for an optimized use of these therapeutic products. The current storage conditions of RBCs differ a little bit depending on countries, but are globally similar (from 5 to 7 weeks at 4 °C in plastic bags allowing gas-exchange, in additive solution mainly composed of glucose, adenine and salts). These non-physiological conditions are not without consequence on erythrocytes, which are known to undergo the so-called storage lesions [1, 2], consisting in metabolism alterations [3-5], oxidative injuries to the cellular content, in particular the protein content [6-8], and modifications of rheological properties [9-11]. A hot debate exists regarding the impact of the storage lesions on post-transfusion adverse outcomes to patients. As the lesions occur and accumulate throughout the storage period, there is a pressure from physicians to be supplied in the freshest possible blood units. This is however not always possible in regard to delivery policy of many blood banks, driven by the first-in first-out rule, to avoid wasting blood products. The storage duration of ECs is questioned [12], and several clinical studies concluded in advising lowering this limit to favor the quality of blood products instead of their availability [13]. Though this is a really defendable point, there is still no clearly stated association of the storage duration with clinical adverse outcomes, which conducted the World Health Organization Blood Regulators Network (WHO BRN) to declare that "*regulatory policies to reduce storage shelf life of red blood cells would be premature*", considering the "*present scientific uncertainty*" and the resulting "*major adverse impact on blood availability*"[2]. Some of the storage lesions are well known, and it appears that many of them are reversible through the transfusion of the blood products. On the contrary, oxidative lesions to RBC proteins are mostly irreversible. If storage lesions are

[2] From the "position statement on reports of inferior clinical outcomes with transfusion of older vs. younger stored red blood cells", available at http://www.who.int/bloodproducts/brn/PositionPaperBloodTransfusionOldYoung7Jul09.pdf?ua=1

susceptible to provoke patient adverse outcomes, it is most likely that irreversible ones would be implied. In this sense, a proteomics investigation of oxidative lesions in RBCs during the banking of ECs has been undertook at the Service Régional Vaudois de Transfusion Sanguine of Lausanne, among a larger project consisting in the assessment of the potential impacts of fabrication processes and storage on blood products quality.

Proteomics relies on powerful tools such as MS, enabling the identification of protein populations, as well as the characterization of PTMs that proteins potentially carry. The major limitation of MS is the 10^4 dynamic range of protein concentrations that can be assessed. In order to access the more comprehensive information possible, sample fractionation is mandatory. Classic approaches consist in gel-free (chromatography based on hydrophobicity, size or charge) and/or gel-based (PAGE according to their MW or pI/MW couple) separation of proteins, followed by a bottom-up MS-based identification (digestion of protein fractions, separation of peptides based on their hydrophobicity, and MS/MS peptide analysis). Based on this standard workflow, different redox proteomics strategies were here set up to investigate 2 hallmarks of protein oxidation, the oxidation of cysteine residues and the carbonylation of proteins, as well as their impact on cellular defense processes.

Results reminder

Oxidation of cysteines

Cysteine oxidation was assessed on RBCs Hb-depleted soluble extracts from 5 ECs stored for 6, 27 and 41 days. Cysteines engaged in reversible oxidative states were reduced and labeled with thiol-reactive IR probes, whereas free cysteines were directly alkylated. Extracts were analyzed in a 2D-DIGE-like approach, and IR spot intensities were quantified by densitometry. This approach permitted to detect spots of interest presenting different evolution of signal intensity over the 3 studied storage periods. The different patterns of evolution indicate proteins differentially affected by the oxidation of cysteine residues. For instance, several spot intensities were constantly increasing, meaning that corresponding proteins were more affected by reversible cysteine oxidation along the RBCs storage. Others spot intensities were decreasing, corresponding to proteins less prone to labeling thus becoming irreversibly oxidized along the

storage period. This study reports for the first time that cysteine oxidation belongs to RBC storage lesion. In addition to highlighting its occurrence, our data suggested a targeted mechanism since not all proteins were affected, and affected proteins showed different susceptibility.

From Coomassie-blue stained 2D gels, spots of interest were excised then proteins were digested, analyzed by LC-MS/MS and classified by GO annotation. Most of identified proteins (56 %) presented specificity towards reversible or irreversible oxidation of cysteines, highlighting a protein-dependent effect. The proportions of molecular functions engaged through modified proteins differed regarding this reversibility, meaning that the oxidation of cysteines is most likely to target protein populations. Among proteins exclusively suffering from irreversible cysteine oxidation, catalytic enzymes (hydrolase, transferase, isomerase) were more represented. Such proteins are thus most likely to present altered structures, and probably functions. Catalytic protein functions are highly involved in cellular processes, such as for instance the glycolysis. Metabolomic data show altered glycolysis during RBCs storage [3, 5]. If substrate depletion is most likely a major issue, oxidative modifications to key enzymes have to be taken into account. In particular, our data highlighted that PGAM4 and TPIS, two enzymes implicated in glycolysis, showed a first increase in reversible oxidation (*i.e.* oxidative stimulation) then a decreasing reversible oxidation (*i.e.* irreversible oxidation or degradation/elimination from the cytoplasm). Additionally, G6PD and TALDO are involved in the PPP and showed constant irreversible oxidation or degradation/elimination. On the contrary, AO and transporter activities appeared more susceptible to reversible cysteine oxidation, suggesting a storage-related increasing stimulation by oxidative conditions, which is not surprising for the former.

Carbonylation of proteins

The carbonylation of proteins during the storage of RBCs was investigated quantitatively by western-blots and qualitatively by an AP-MS approach followed by GO annotation. To better assess this modification, weekly collected RBCs from 3 ECs were efficiently fractionated at the subcellular level. Soluble Hb, Hb-depleted cytosol, integral membrane proteins and cytoskeleton fractions were thus investigated for protein carbonyl quantitation through chemical derivatization and immunodetection of derivatized proteins. This study allowed to characterize

a time-dependent and compartment-dependent evolution of protein carbonylation during RBCs storage, with a particularly interesting membrane-related end-storage increase for both integral and cytoskeleton proteins, starting from 3 and 4 weeks of storage, respectively. Regarding the integral membrane fraction, a decrease in carbonylation was observed at the very end of the storage, suggesting a loss of the damaged material. Soluble fractions revealed a starting decreasing pattern, stabilized after 2 weeks, which let thinking of a processing of soluble carbonylated content in the beginning of the storage.

The proteomic analysis of proteins affected by carbonylation implied a chemical derivatization with biotin in order to purify carbonylated proteins before LC-MS/MS analysis. In the Hb-depleted soluble fraction, a qualitative change in targeted proteins was demonstrated along the storage. In particular, AO activity-bearing carbonylated proteins showed a higher proportion at day 26, further decreased at day 41, suggesting a particular occurrence of carbonylation in this protein population during the first half of storage, afterwards these proteins should be processed, making them less detectable from soluble fraction. From what is known regarding cellular processes of proteins in RBCs, this can mean either membrane recruitment, proteolytic degradation by the proteasome, or elimination through the release of microvesicles. Of interest are numerous carbonylated enzymes involved in glycolysis (G3PD, ENOA and PGM2, mainly carbonylated during the first half of storage) and in the PPP (6-PGD, RPIA, TALDO, and TKT, mainly carbonylated during the second half of the storage period). These results on glycolytic enzymes are in accordance with cysteine oxidation ones, in the sense that key proteins of RBCs metabolism are oxidatively altered during the storage period. Moreover, regarding enzymes out of the PPP, their absence in the soluble carbonylome at the end of the storage and their decreasing cysteine-labeling intensity at day 41 suggest high oxidative injuries during the 4 first weeks of storage and either a membrane recruitment or degradation/elimination at the end of RBCs banking. Regarding enzymes from the PPP, they appear to suffer from irreversible cysteine oxidation and were identified in the soluble carbonylome mainly starting from the 4 weeks limit, suggesting that from that storage time they cannot be degraded. Data from Zolla's group suggest a recruitment of the PPP after 2 weeks of storage [5], which does not seem coherent with the present data, where PPP enzymes were found damaged during the second half of

storage.

As for the membrane carbonylation, changes were mostly quantitative, with increasing copies of carbonylated proteins (only 20 different proteins) along the storage, with 55 % of identified proteins being common to the three storage durations.

Inhibition of the P20S activity

Future of oxidized proteins in living cells is quite clear. What becomes useless is degraded. Proteasome is the cellular machinery allowing the proteolysis of oxidized proteins among other denatured proteins [14]. However, it is known that oxidative injuries can lead to protein crosslinking. As we showed that stored RBCs are an environment prone to protein oxidation, a particular interest was put on the effective proteolysis activity during ECs banking. Our data show that the P20S-driven proteolysis is affected by a 40 % loss of its activity at day 41 in comparison to samples from day 6 and day 27. Here again, it seems that after around 4 weeks of storage, a major cellular process suffers from the conditions offered by the storage of RBCs. Storage-related activity modulation could be associated with the storage-related increase in oxidative injuries to proteins. Characterization of the soluble carbonylome revealed altered sub-units of the proteasome complexes, as shown in Part 3, table 2 (more sub-units of the P20S complex were identified in the soluble carbonylome, but were not validated with our criteria, thus do not appear in this table). In addition, oxidized proteins tend to crosslink, and are recognized by P20S complexes [15]. We thus investigated the potential inhibitory effect of increasing quantities of exogenous oxidized proteins on the P20S activity in fresh RBCs samples. A dose-dependent and protein-dependent inhibitory effect was demonstrated through our experiments, highlighting an interesting higher induced inhibition by oxidized Hb compared to oxidized BSA.

Release of microvesicles

All along the storage period, RBCs are known to release increasing amounts of MVs [16], with an intense exponential release starting from 4 weeks of storage. *In vivo* as in stored ECs, heterogeneous populations of RBCs are encountered regarding the age of cells. Whether all cells or only old ones participate to the MV release is unknown. As a consequence, one cannot

state if cytoskeleton destabilization through hemichromes binding originates the release of MVs. Hemichromes binding to membrane [17] is likely to induce conformational changes of band 3, creating a surface neo-epitope [18] recognized by naturally occurring anti-band 3 auto-antibodies [19, 20], favorizing the *in vivo* clearance of RBCs. Moreover, storage-released MVs have been found enriched in such altered band 3. As a consequence, it seems likely that hemichromes binding to RBCs membrane occurs before the massive MVs release. Pushing further, one can hypothesize a signal role of hemichromes binding towards microvesiculation, through the destabilization of the membrane-to-cytoskeleton association. This way to eliminate such aging marker makes microvesiculation of potential mechanism of defense.

Conclusions – an oxidative pathway model of the RBCs storage

In addition to a storage lesion, oxidative modifications, and particularly the oxidation of cysteines is known to be involved in multiple cellular processes, through oxidation-reduction cycles. AO enzymes are the best example. Soluble AO were shown to be less prone to irreversible cysteine oxidation during storage, suggesting that their active site would be preserved, allowing RBCs to fight against ROS, as shown in Figure 38, step1. However, they present a higher carbonylation status by the first half of the storage period. This correlates with the double incidence (cellular processes and protein lesions) of oxidation in the case of RBCs storage. The global quantitative decrease in soluble carbonylated proteins should thus be related to a preserved AO activity up to the first 2 weeks of storage (Figure 38, step 1). Up to day 26, AO proteins appeared to cumulate oxidative injuries through carbonylation. However, the proportion of this protein family is lowered from day 26 to day 41. Protein oxidation is known to induce recognition and degradation by 20S proteasome complexes (Figure 38, step 2), which would fit with the observed decrease. Increasing oxidation of AO defenses, though apparently not altering their active sites and beyond making them prone to proteolysis is likely to change them into proteasome inhibitors, as suggested by the protein oxidation dose-dependent inhibitory effect observed on P20S activity.

In summary, efficient AO defenses, among other proteins, would accumulate oxidative injuries until around 4 weeks of storage, making them recognized by P20S complexes to either being degraded (Figure 38, step 2) or inhibiting the proteasomal activity (Figure 38, step 3). AO defenses would thus decrease so low that they would not be able to counterbalance oxidants, allowing overoxidation of proteins which cannot be taken over by the proteolysis machinery anymore, as enzymes from the PPP seem to be. Protein overoxidation is known to induce the formation of aggregates. Regarding Hb, its aggregated forms termed hemichromes are known to bind band 3 on its cytoplasmic domain (Figure 38, step 4). Band 3 proteins are the loci of membrane-to-cytoskeleton binding, and also of multiprotein complexes. In particular, proteins belonging to the glycolysis chain are found in band 3 complexes. The binding of hemichromes is likely to affect the structure of the cytoplasmic domain of band 3, thus displacing these proteins and

potentially inducing destabilization of cytoskeleton and altering glycolytic functions of RBCs. Hemichromes like Hb are capable of autoxidation, which can participate to oxidative injuries that we have shown to accumulate on cytoskeleton and membrane proteins (Figure 38, step 5), as well as on glycolysis and PPP enzymes. The hemichromes binding and further oxidation of the cytoskeleton and membrane environment might be a signal for high release of aging markers and other modified proteins by microvesiculation (Figure 38, step 6).

Contrary to several clinical studies on blood age-related transfusion-linked adverse outcomes, and some research studies on RBCs storage lesions and altered metabolism, tending to show a 14-day storage limit after which RBCs would not be suitable for transfusion, our results suggest that oxidative stress-linked alterations of stored RBCs rather tend to a longer limit of 4 weeks. In Lausanne, statistics of transfusion at the CHUV (see Part 1) showed that half ECs are transfused during the 2 first weeks of storage, and that around 90 % are transfused during the 4 first weeks. As a consequence, it seems that, in most cases, transfused RBCs are healthy enough to ensure the transfusion goal of increasing tissue oxygenation capacities. However, statistics had also shown a particular difference for transfusions of B and AB rare blood groups, particularly true for the AB^+ blood group. These groups revealed a mean higher transfusion abundance of ECs stored for 6 weeks. For these particular cases, our observations thus tend to be in disfavor of a more than 4-week EC storage duration, and further investigations should be conducted in regard to the effect of storage duration on clinical adverse outcomes to transfusion recipients of B and AB blood groups. According to storage conditions (in particular the additive solution) and policies of ECs delivery, these conclusions may be modified. At the time of a patient-specific medicine, the storage of blood products for transfusion might become recipient-dependent as well.

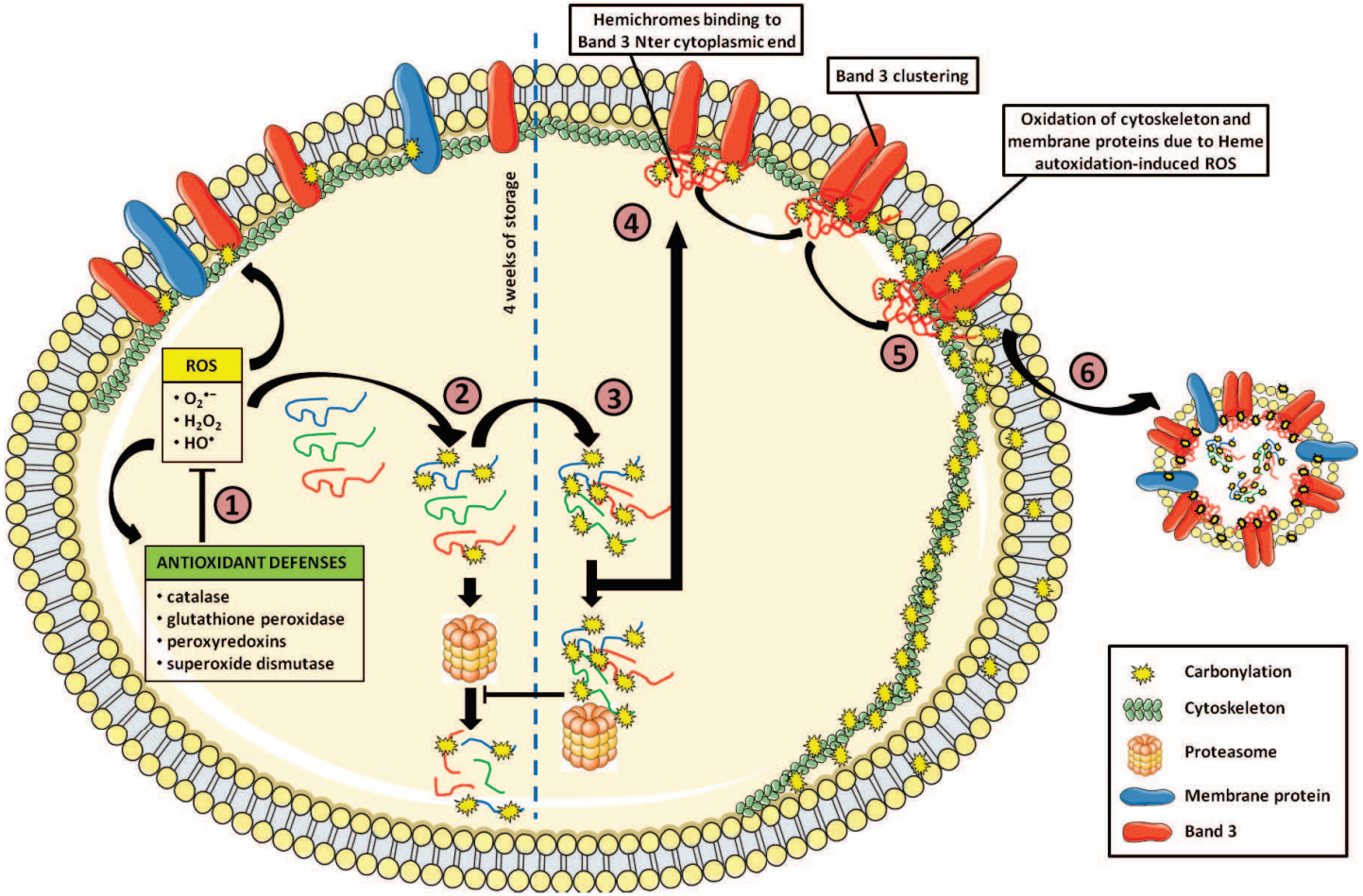


Figure 38: Oxidative pathway model of RBCs storage. Step 1: RBCs antioxidant defenses prevent most oxidative damages by reducing ROS into less reactive intermediates, or being oxidized themselves, followed by a recycling mechanism. After a certain storage period, these defenses are exceeded by the oxidative stress and protein oxidation occurs at both cytosolic and membrane levels. Step 2: oxidized proteins are recognized by the 20S proteasome complexes through exposure of hydrophobic moieties. Antioxidant defenses are oxidized as well, and are susceptible to proteolysis, allowing for overoxidation of RBCs content. Step 3: surrounding 4 weeks of storage, overoxidized proteins crosslink and cannot be degraded anymore, inhibiting the proteolysis of fairly oxidized as well as other damaged proteins. Step 4: oxidized Hb aggregates as hemichromes and binds to membrane band 3 protein, modifying its conformation and potentially altering its association with cytoskeleton and displacing bound glycolytic enzymes. Step 5: hemichromes autoxidation produces ROS which oxidize cytoskeleton and membrane proteins. Step 6: exponential phase of the MVs release, allowing the elimination of RBCs aging markers such as altered band 3 and externalized phosphatidylserine.

Perspectives

The age of transfused blood products, particularly of ECs, is a currently highly debated question in the transfusion medicine field. As introduced in Part 1, several clinical studies tend to correlate “old blood” transfusion with recipients adverse outcomes. But what is an “old blood”? Can we really define precise storage duration after which, despite suitable transfusion prognostics (storage hemolysis and *in vivo* 24-hour recovery), a blood product would be undoubtedly harmful for its recipient? In fact, clinical studies cannot delimit such single storage duration. There are several obvious reasons for that. First, an EC is not a globally defined blood product. Depending on where you live, the production way of this therapeutic product will change. From the blood collection to the delivery of an EC unit, many parameters make it different depending on countries. If one of these parameters should be highlighted, it would be the additive solution used to preserve RBCs during their non-physiological cold storage. Formulations of diverse AS used worldwide seem similar in term of components, but some subtle changes appear to significantly impact the storage of RBCs, as discussed in Part 1. The energy metabolism is a key point for RBCs maintenance, bringing the required energy for diverse cellular processes. It is currently being investigated during ECs storage by several groups, and it appeared to be differentially impacted, depending on the AS. It thus seems difficult to attribute clinical adverse outcomes to a given storage duration, while in fact all products of this age are not identical. Another reason making difficult the establishment of a storage limit for a guaranteed successful transfusion procedure is the health status of recipients. Will a same product similarly affect patients suffering from different unhealthy conditions? Deeping further, will a same product similarly affect two patients presenting the same hematologic disorder, if one of them is young and the other old? What if one is a man and the other a woman? Most clinical investigations regarding age of ECs focused on a particular situation, for instance patients from a given type of surgery, and individual characteristics are being taken into account too. But until now, it seems to be a very difficult task to undoubtedly associate blood storage duration to transfusion-related adverse issues.

Increasing ECs storage duration brings its batch of lesions to RBCs. If clinical studies tend to find a storage limit before which these lesions will not be hazardous for the transfusion recipients, issues regarding blood supply have to be considered. In the other side of the recipients, scientists are assessing storage lesions in order to bring comprehensive data on potential post-transfusion complications. Moreover, new ways to store blood products, avoiding the occurrence of these lesions are being developed and are under investigation. New additive solutions, pre-transfusion procedures and special storage conditions are being investigated, and gave interesting results. In particular, oxygen-depleted storage environment and addition of antioxidants to additive solutions seem promising. Efforts should be put on such strategies, not necessarily aiming at prolonging storage durations based on RBCs stability during storage and after transfusion, but to understand what happens in stored ECs, to try preventing or correcting lesions, maybe to find new markers of RBCs quality, and most certainly to supply physicians and more importantly patients, with blood products of the highest quality.

In this sense, my PhD thesis gave access to new comprehensive data regarding the oxidative character of the RBCs storage. Targeted cellular processes were highlighted, opening investigation routes towards further developments of RBCs storage conditions. Additionally to improving global knowledge in the domain of transfusion medicine and the research on RBCs storage lesions, my work will be at the basis of further investigations that our research team will conduct. The present established data concerning currently used storage conditions will serve as reference for ongoing studies in our institution. In particular, oxidation-related data will be of high importance regarding our current experiments on oxygen-depleted RBCs storage and investigations of global metabolism, as well as for upcoming studies on additive antioxidants. Moreover, the biochemical and proteomic methods developed will be useful in the scope of another PhD thesis assessing the oxidative effects of pathogen reduction technologies on platelet concentrates.

Short-term perspective will focus on the characterization of RBCs membrane proteins presenting storage-induced oxidation of cysteines. In particular, it would be interesting to correlate membrane-bound oxidized proteins such as Hb, enzymes from the energy

metabolism, or cytoskeleton components with the proposed oxidative pathway model. This work is part of a global strategy to improve RBCs storage, where the next steps will be based on this research.

References

1. Hess, J.R., *Red cell changes during storage.* Transfus Apher Sci, 2010. **43**(1): p. 51-9.
2. D'Alessandro, A., et al., *An update on red blood cell storage lesions, as gleaned through biochemistry and omics technologies.* Transfusion, 2014.
3. Roback, J.D., et al., *Metabolomics of ADSOL (AS-1) red blood cell storage.* Transfus Med Rev, 2014. **28**(2): p. 41-55.
4. Yoshida, T., et al., *The effects of additive solution pH and metabolic rejuvenation on anaerobic storage of red cells.* Transfusion, 2008. **48**(10): p. 2096-105.
5. D'Alessandro, A., et al., *Time-course investigation of SAGM-stored leukocyte-filtered red bood cell concentrates: from metabolism to proteomics.* Haematologica, 2012. **97**(1): p. 107-15.
6. Dumaswala, U.J., et al., *Protein and lipid oxidation of banked human erythrocytes: role of glutathione.* Free Radic Biol Med, 1999. **27**(9-10): p. 1041-9.
7. Kriebardis, A.G., et al., *Membrane protein carbonylation in non-leukodepleted CPDA-preserved red blood cells.* Blood Cells Mol Dis, 2006. **36**(2): p. 279-82.
8. Kriebardis, A.G., et al., *Progressive oxidation of cytoskeletal proteins and accumulation of denatured hemoglobin in stored red cells.* J Cell Mol Med, 2007. **11**(1): p. 148-55.
9. Berezina, T.L., et al., *Influence of storage on red blood cell rheological properties.* J Surg Res, 2002. **102**(1): p. 6-12.
10. Frank, S.M., et al., *Decreased erythrocyte deformability after transfusion and the effects of erythrocyte storage duration.* Anesth Analg, 2013. **116**(5): p. 975-81.
11. Hovav, T., et al., *Alteration of red cell aggregability and shape during blood storage.* Transfusion, 1999. **39**(3): p. 277-81.
12. van de Watering, L.M., *Age of blood: does older blood yield poorer outcomes?* Curr Opin Hematol, 2013. **20**(6): p. 526-32.
13. Koch, C.G., et al., *Duration of red-cell storage and complications after cardiac surgery.* N Engl J Med, 2008. **358**(12): p. 1229-39.
14. Ciechanover, A., A. Orian, and A.L. Schwartz, *Ubiquitin-mediated proteolysis: biological regulation via destruction.* Bioessays, 2000. **22**(5): p. 442-51.
15. Pacifici, R.E., Y. Kono, and K.J. Davies, *Hydrophobicity as the signal for selective degradation of hydroxyl radical-modified hemoglobin by the multicatalytic proteinase complex, proteasome.* J Biol Chem, 1993. **268**(21): p. 15405-11.
16. Rubin, O., et al., *Microparticles in stored red blood cells: an approach using flow cytometry and proteomic tools.* Vox Sang, 2008. **95**(4): p. 288-97.
17. Waugh, S.M. and P.S. Low, *Hemichrome binding to band 3: nucleation of Heinz bodies on the erythrocyte membrane.* Biochemistry, 1985. **24**(1): p. 34-9.
18. Kay, M.M., et al., *Senescent cell antigen is immunologically related to band 3.* Proc Natl Acad Sci U S A, 1983. **80**(6): p. 1631-5.
19. Hornig, R. and H.U. Lutz, *Band 3 protein clustering on human erythrocytes promotes binding of naturally occurring anti-band 3 and anti-spectrin antibodies.* Exp Gerontol, 2000. **35**(8): p. 1025-44.
20. Lutz, H.U. and G. Stringaro-Wipf, *Senescent red cell-bound IgG is attached to band 3 protein.* Biomed Biochim Acta, 1983. **42**(11-12): p. S117-21.

ANNEX A – Details on ECs used for experiments

EC reference	Donor sexe	Donor age	Blood group	Study
EC1	Female	31	A+	1, 3, 4, 5
EC2	Male	72	O+	1, 3, 4, 5
EC3	Female	20	O+	1, 3, 4, 5
EC4	Female	61	A+	1, 3
EC5	Male	55	A-	1, 3
EC6	Female	64	A+	2
EC7	Male	59	B+	2
EC8	Female	19	A+	2
EC9	Female	21	O+	6
EC10	Female	25	B+	6
EC11	Female	19	O+	6
EC12	Female	21	A+	6
EC13	Male	44	A+	6
EC14	Female	53	O+	6

Table 1. Donor details of erythrocyte concentrates used for the study of cysteine oxidation. Study 1 stands for the detection of cysteine oxidation (PART 2, chapter one), study 2 for the quantitation of protein carbonylation (PART 2, chapter two), study 3 for the identification of proteins presenting modifications of cysteine oxidation during the storage (PART 3, chapter one), study 4 for the identification of carbonylated proteins along the storage period (PART 3, chapter two), study 5 for the characterization of P20S activity during storage and its behavior in oxidative conditions and study 6 for the determination of loss of carbonylated proteins in microvesicles.

ANNEX B – Characterization of protein extracts

Material and methods

Protein extracts were analyzed by SDS-PAGE. 15 µg of proteins of each extract and 3 µL of BenchMark Protein Ladder were loaded on precast Mini-PROTEAN TGX gels, 4-15 % (BIO-RAD, USA). Proteins were stained with Coomassie Brilliant Blue R250 and gel was destained so as to obtain the optimum contrast. Extracts were prepared as described in Part 1 Chapter 2, from erythrocytes stored for 8 days post-collection. Moreover, membrane proteins were extracted under different conditions. A 1.5 mL-tube containing washed membranes was split in 2, and centrifuged at 18000 *g* for 30 minutes at 4 °C. From the first half, the total membrane extract was obtained by solubilizing the membrane proteins with DC buffer. The second pellet was resuspended in DDM buffer and centrifuged as before. The supernatant (DDM membrane extract) was stored at 4 °C and the pellet was washed three times with DDM buffer (only the first wash was kept for SDS-PAGE analysis). Then, the pellet was solubilized in DC buffer. After a final centrifugation, the supernatant (DC membrane extract) was stored at 4 °C for further analyses.

Membrane protein identification was achieved on a liquid chromatography-mass spectrometer apparatus (Agilent 1100 series LC/MSD trap) from Agilent Technologies (Santa Clara, CA, USA) after membrane protein separation on SDS-PAGE. This identification was performed to ensure that the two different membrane extracts obtained correspond, as thought, to the integral and the peripheral (cytoskeleton) membrane fractions. As for the two soluble fractions, SDS-PAGE separation was sufficient to characterize the depletion of one major protein (*i.e.* Hb).

RBC soluble fractions

Proteins from RBCs were fractionated in different extracts, which allowed the analysis of protein carbonylation within different compartments. Figure 39 shows the protein content of each sample extract (lanes 1 to 5). As already reported, the Hb-depletion clearly exhibits the gain in dynamic range [1]. The depletion removed 95 % of the Hb from the cytoplasmic extract (data not shown) and Hb fraction contains a dim quantity of other proteins. Hence, the carbonylation analyses will focus on soluble Hb and on Hb-depleted soluble extracts.

RBC membrane fractions

Prior to membrane extractions, it is of importance to note that, after the washing steps, membranes from end-storage ECs remained redder than membranes from short-storage ones, indicating the presence of more insoluble Hb-based material.

Lanes 4 and 5 (Figure 39) represent the two membrane protein extracts following the sequential extraction with DDM and DC buffers. The lane patterns exhibit a clear difference, which indicates various types of proteins solubilized. In order to point out the differences, RBC membranes were treated either directly with DC buffer (total membrane fraction) or with DDM buffer, followed by three steps of washing with the same buffer and a final extraction with DC buffer (see Figure 39, lanes 7 to 10 respectively). As expected, the washes (only the first one was shown) have the same pattern as DDM membrane extract, and the differences between DDM and DC extracts are highlighted. Five main differences were observed: spectrin region above 220 kDa, band 3 around 90 kDa, proteins 4.1 and 4.2 below the band 3, and actin and stomatin regions at 45 and 33 kDa, respectively (see lanes 8 and 10 in Figure 39 for highlighted differences).

Integral membrane proteins, *e.g.* band 3, protein 4.2, stomatin, were more abundant in DDM membrane extract. Band 3 is a transmembrane protein embedded within the lipid bilayer [2-4], protein 4.2 is a peripheral membrane protein connecting band 3 and Rhesus complexes [3, 5], and stomatin is found in lipid rafts as shown by Salzer and Prohaska [6]. They have shown that stomatin has a low solubility in Triton X-100 buffer because of its association with lipid raft. In the present study, stomatin was however extracted with the DDM buffer, a detergent similar to Triton X-100. This is explained by the presence of aminocaproic acid in the DDM buffer, which has been reported to allow the extraction of membrane-embedded complexes at high salt concentrations [7]. On the other hand, Crepaldi Domingues *et al.* have shown that stomatin is recovered in both soluble and insoluble fractions after Triton X-100 extraction [8]. Various parameters may influence the membrane protein extraction, such as the detergent-to-protein ratio or the existence of microdomains [8].

As for DC membrane extract, obtained with an ionic detergent, it contained proteins stemming

from the peripheral membrane cytoskeleton, such as α- and β-spectrin, protein 4.1 and actin. In particular, protein 4.1 is implied in the junctional or 4.1R complex [9, 10]. Unlike protein 4.2, protein 4.1 interacts directly with the cytoskeleton [9].

Hence, this sequential membrane protein extraction enables the partial separation of membrane proteins and cytoskeleton, beyond the cytosol/membrane sample fractionation.

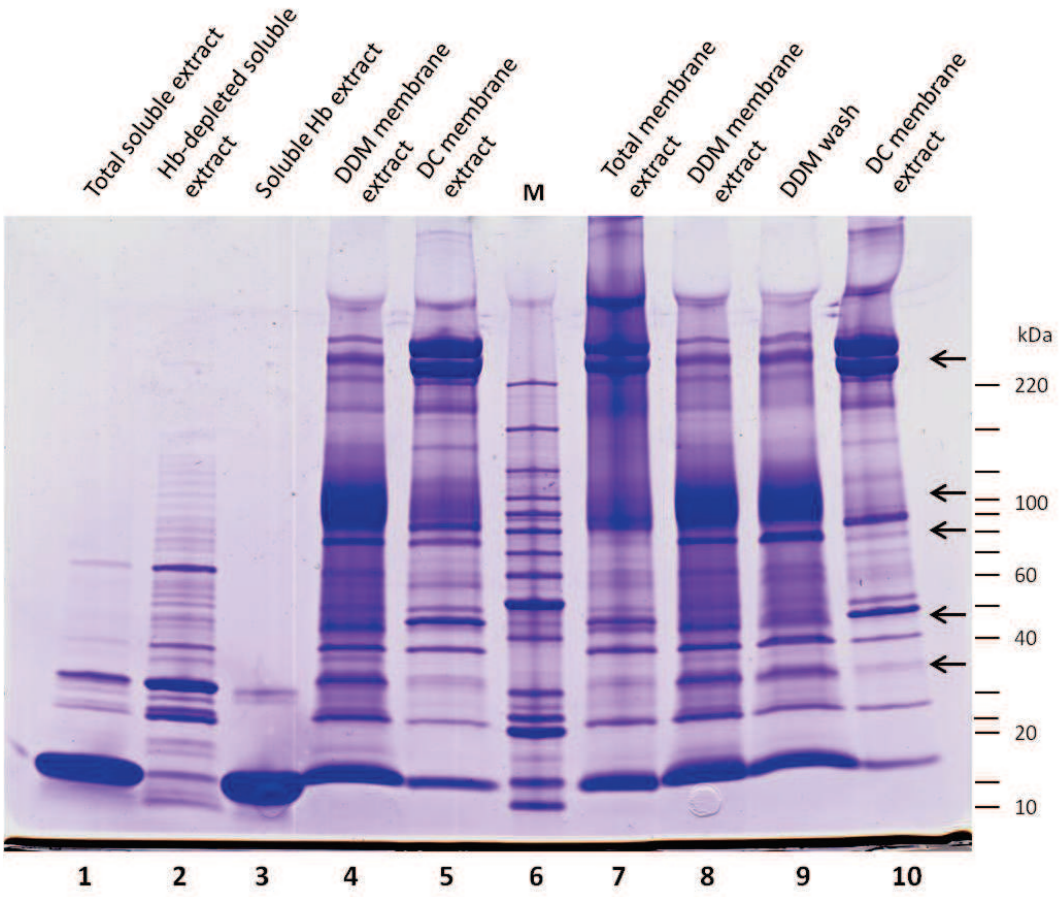


Figure 39 SDS-PAGE of RBC subcellular fractionation. SDS-PAGE of the different sample extracts. 15 µg of proteins were loaded on each lane. Left side of the MW marker: typical sample extracts used for quantitation of protein carbonylation. The depletion of Hb (lanes 1 to 3) and the sequential solubilization of membrane proteins (lanes 4 and 5) allowed the fractionation of RBC protein samples and the analysis of proteins of lower abundance. Right side of the MW marker: characterization of membrane extracts. The first extraction with DDM (lane 8), a non-ionic detergent, mainly extracted the membrane proteins whereas the last extraction with DC (lane 10), an ionic detergent, mainly extracted the cytoskeleton proteins. Arrows on the right point out the main differences characterizing these two extracts (see text for details).

References

1. Ringrose, J.H., et al., *Highly efficient depletion strategy for the two most abundant erythrocyte soluble proteins improves proteome coverage dramatically.* J Proteome Res, 2008. **7**(7): p. 3060-3.
2. Tanner, M.J., *The structure and function of band 3 (AE1): recent developments (review).* Mol Membr Biol, 1997. **14**(4): p. 155-65.
3. Bruce, L.J., et al., *A band 3-based macrocomplex of integral and peripheral proteins in the RBC membrane.* Blood, 2003. **101**(10): p. 4180-8.
4. Burton, N.M. and L.J. Bruce, *Modelling the structure of the red cell membrane.* Biochem Cell Biol, 2011. **89**(2): p. 200-15.
5. Satchwell, T.J., et al., *Protein 4.2: a complex linker.* Blood Cells Mol Dis, 2009. **42**(3): p. 201-10.
6. Salzer, U. and R. Prohaska, *Stomatin, flotillin-1, and flotillin-2 are major integral proteins of erythrocyte lipid rafts.* Blood, 2001. **97**(4): p. 1141-3.
7. Nijtmans, L.G., N.S. Henderson, and I.J. Holt, *Blue Native electrophoresis to study mitochondrial and other protein complexes.* Methods, 2002. **26**(4): p. 327-34.
8. Crepaldi Domingues, C., et al., *Resistance of human erythrocyte membranes to Triton X-100 and C12E8.* J Membr Biol, 2009. **227**(1): p. 39-48.
9. Salomao, M., et al., *Protein 4.1R-dependent multiprotein complex: new insights into the structural organization of the red blood cell membrane.* Proc Natl Acad Sci U S A, 2008. **105**(23): p. 8026-31.
10. van den Akker, E., et al., *Band 3 multiprotein complexes in the red cell membrane; of mice and men.* Blood Cells Mol Dis, 2010. **45**(1): p. 1-8.

ANNEX C – Depletion of oxidized hemoglobin

A sample of RBCs was taken from an EC stored for one day. Cells were washed with 0.9 % NaCl then lysed by hypotonic shock (incubation with 2 cell volumes of 0.1 X PBS under agitation at 4 °C during 1 hour). Membranes were pelleted and the soluble protein extract was recovered from the supernatant. One mL of soluble extract was loaded in the chromatographic system for Hb depletion, and the UV signal for protein detection was monitored and is shown in Figure 1.

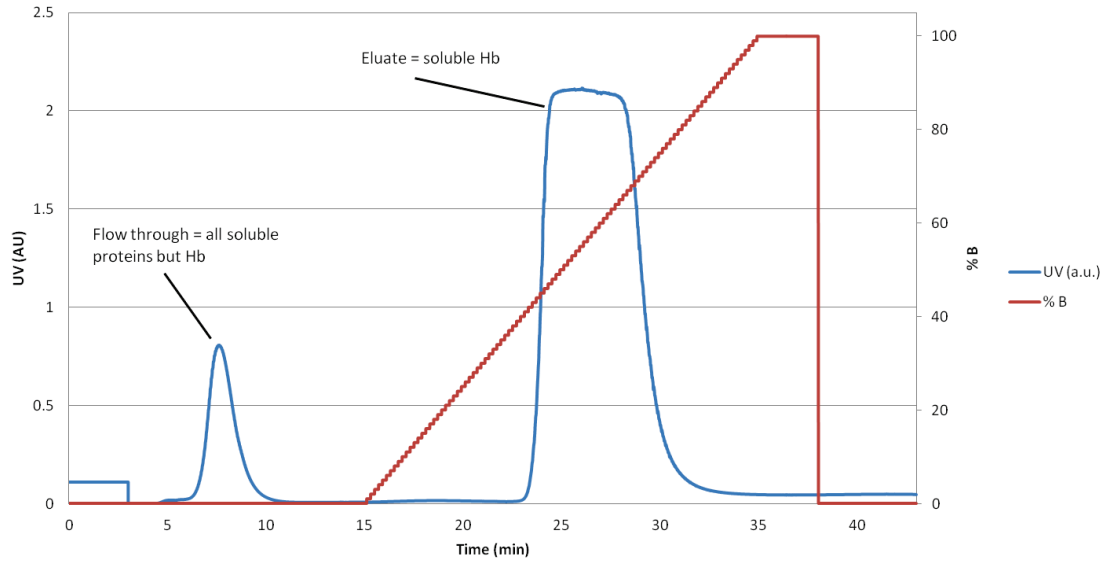


Figure 40. Run of hemoglobin depletion from a RBC soluble protein extract. The first peak before the beginning of the elution gradient is the flow through, corresponding to unbound proteins (all proteins except hemoglobin). The second peak is eluted with buffer B, and corresponds to the hemoglobin fraction.

The eluate consisting of the Hb was collected as a single fraction. The Hb fraction was splited in two. One half was conserved as is, and the other half was submitted to chemical oxidation by incubation with one volume of 2X oxidation solution (50 mM HEPES pH 7.2, 50 mM ascorbic acid, 200 µM $FeCl_3$) under agitation during 5 h at 37 °C and in the dark. Oxidized Hb was then dialyzed overnight at 4 °C against a solution of 50 mM HEPES pH 7.2 buffer containing 1mM EDTA. Hb preparations were both submitted to Hb-depletion.

Untreated Hb sample is entirely retained in the IMAC column, as shown in Figure 2. UV signal only detect proteins in the eluate. As for the over-oxidized Hb sample, the majority of the sample went through the depletion column, and high protein content was detected in the flow through, as shown in Figure 3. A little part of proteins was however retained by the solid phase,

indicating an incomplete oxidation of the sample. Surprisingly, the flow through containing nearly all hemoglobin was colorless, whereas the eluate, which contained few proteins, was reddish. The red color of hemoglobin is due to the iron oxidation occurring through oxygen binding. The hard oxidative conditions to which was submitted the Hb sample may certainly alter the heme core of Hb, originating the loss of coloration. Moreover, the binding site of Hb to nickel (His-2 residue of the β globin chains, see reference [1]) is probably altered too, so that highly oxidized Hb does not bind the IMAC column anymore.

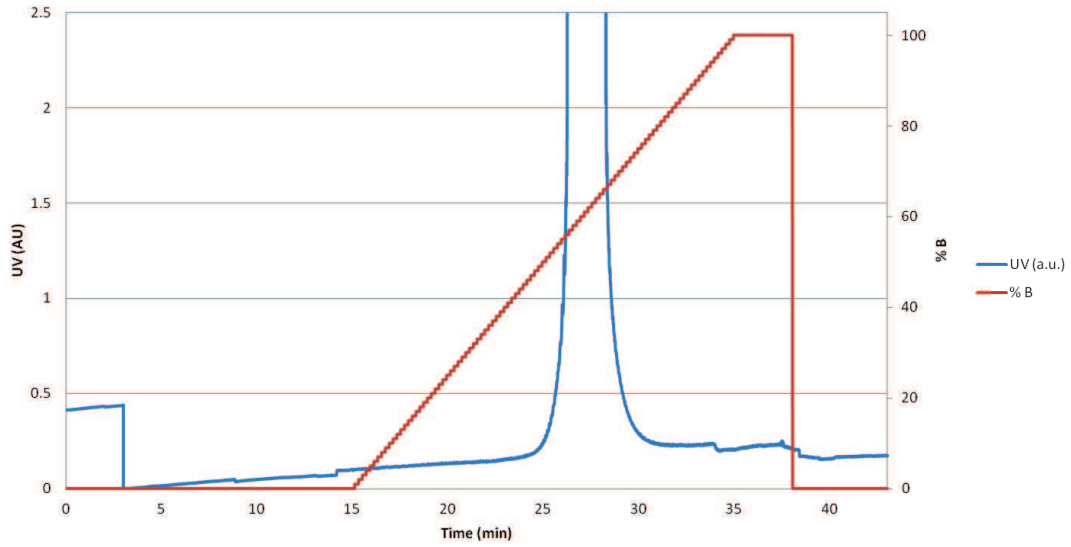


Figure 41. Run of hemoglobin depletion from the untreated Hb fraction. All proteins are detected in the eluate.

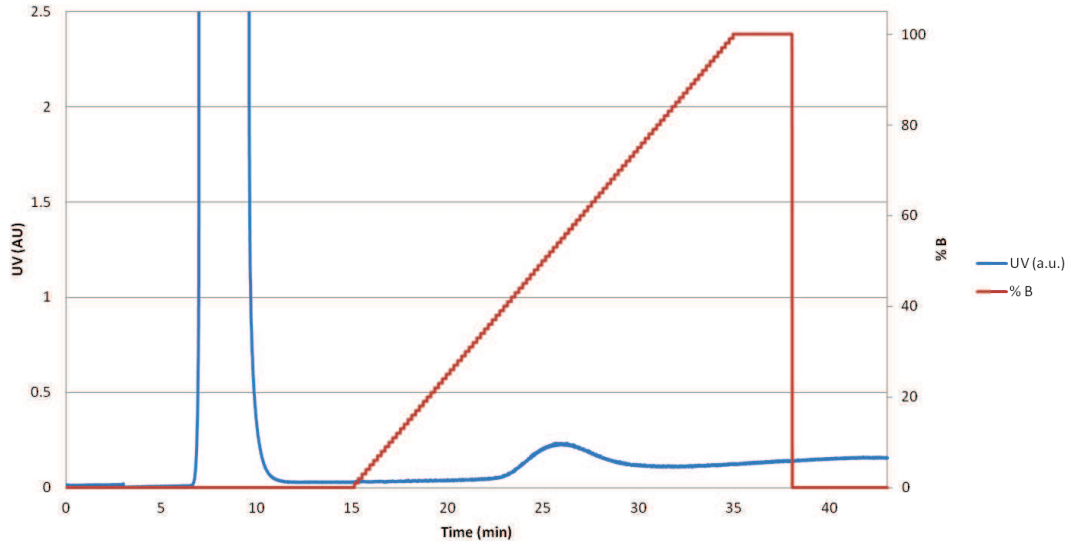


Figure 42. Run of hemoglobin depletion from the oxidized Hb fraction. The majority of proteins are detected in the flow through and a small part in the eluate.

Reference

1. Levine, J., et al., *Identification of a nickel(II) binding site on hemoglobin which confers susceptibility to oxidative deamination and intramolecular cross-linking.* J Biol Chem, 1998. **273**(21): p. 13037-46.

DELOBEL JULIEN – PROTEOMIC SCIENTIST

Route de la Verreyre 2 | 1088, Ropraz (Switzerland) | +41 (0)78.695.07.08 | delobel.julien@free.fr
Nationality : French | Date of birth : 7th February, 1987 | marital status : single
LinkedIn profile | ResearchGate profile

EDUCATION

- 01/2010-12/2014 : **PhD in Life Sciences**, University of Lausanne, **Switzerland**
- 09/2007-09/2009 : **Professional Master** in Sciences and Technologies, mention Biology and Biotechnologies, specialized in Proteomics, Lille 1 University – Science and Technology, USTL, Villeneuve d'Ascq, **France**
- 09/2006-06/2007 : **Bachelor** in Sciences and Technologies, specialized in genomics and proteomics, USTL, **France**
- 09/2004-06/2006 : **Technical University Diploma**, specialized in Biological and Biochemical Analyses, USTL, IUT "A", **France**
- 2004 : **High school diploma**, scientific section, specialized in Life and Earth Sciences, Lycée Arthur Rimbaud, Sin-le-Noble, **France**

WORK EXPERIENCES

PhD in Life Sciences 04/01/2010 — 31/12/2014

Proteomics of oxidative damages undergone by red blood cells during blood banking of erythrocyte concentrates

Supervisor: Prof. Jean-Daniel Tissot

Service Régional Vaudois de Transfusion Sanguine (SRTS-VD), Route de la Corniche 2, 1066 Epalinges, **Switzerland**

- Management of blood samples
- Subcellular fractionation and protein extraction/purification/separation
- Derivatization (hydrazine-based reagents), labeling (maleimide-based probes) and purification (biotin-derivatives) of oxidatively modified proteins
- Detection, quantitation and identification of oxidatively damaged proteins
- Fluorescence-based kinetics for enzymes activity determination (esterase and 20S proteasome activities)
- Supervision of student and technician

Master 2 training period (mandatory) 02/02/2009 — 31/07/2009

Effect of formaldehyde-induced protein cross-linking on the purification of cytoplasmic and nuclear proteasome complexes from the human leukemia cell line U937

Supervisor: Dr. Marie-Pierre Bousquet-Dubouch

Team of Proteomics and Mass Spectrometry of Biomolecules, IPBS, Toulouse, **France**

- Human cell culture
- Sepharose-binding of monoclonal antibodies
- Purification, characterization and identification of cytoplasmic and nuclear proteasome complexes

Master 2 internship (optional) 01/09/2008 — 01/12/2008

Development of liquid ionic matrices for MALDI-MS and MALDI-Imaging

Supervisor: Dr. Julien Franck

Laboratory of fundamental and applied biological mass spectrometry, USTL, **France**

- Preparation of liquid ionic matrices and automated deposition testing (micro-sprayer and micro-spotter)
- Testing of automated matrix deposition devices (micro-sprayer and micro-spotter)

Master 1 training period (mandatory) 16/03/2008 — 16/08/2008

2D gel analysis of in-house engineered plasmid-induced over-expression of Vitamin B12 biosynthesis enzymes in E.coli

Supervisor : Dr. Evelyne Deery

Biochemistry group, Department of Biosciences, University of Kent, Canterbury, **United Kingdom**

- Bacterial transformation and culture
- 2D electrophoresis (set-up of the methodology) – MALDI-MS identification

Training period for the Technical University Diploma (mandatory) 10/04/2006 — 16/06/2006

Transcriptomic analysis of enzymes involved in protein glycosylation in Toxoplasma gondii

Supervisor : Prof. Stanislas Tomavo

Team of molecular parasitology, Unit of structural and functional glycobiology, USTL, **France**

- Primer design, RT-PCR, agarose gel electrophoresis of amplification products
- Cloning and expression of proteins of interest in bacterial systems

SKILLS

- Technical skills : bacterial and cellular culture, PCR, protein derivatization, purification of protein complexes, kinetic for enzyme activity determination, flow cytometry, 1D and 2D electrophoresis, Western-Blot, liquid chromatography (affinity, gel-filtration, reversed phase), mass spectrometry (MALDI-TOF, MALDI TOF-LIFT, LTQ-Orbitrap, ESI-ion trap, and associated softwares), MALDI imaging, MS/MS label-free quantitation, ETD fragmentation
- Informatics: MS office, bio-informatics (ImageMaster, Expasy, Blast2GO), proteomics and mass-spectrometry softwares (Chromeleon for Thermo HPLC control, FlexControl and FlexAnalysis for Bruker MALDI, HyStar and trapControl for Bruker ion trap, ProteinScape for project management and protein database searching), ChemBioDraw for molecular drawing, R programming for statistics
- Soft skills: **project management** (supervision of undergraduates and delegation of work to technicians), **teamwork** (involvement in several collaborative projects), **teaching** of students and technicians, written and oral **communication** skills (working instructions, research reports, lab meetings, journal clubs), **scientific popularization** (job discovery days and “child at work” days)

LANGUAGES

- French (mother tongue)
- English (spoken, read and written, B2 level)
- Spanish (school level)

INTERESTS

- Team sports (football, volleyball, rugby)
- Scuba diving and snorkeling
- Japanimation
- Reading manga and comic books
- Trading card games

REFERENCES

Prof. Jean-Daniel Tissot | jean-daniel.tissot@mavietonsang.ch | +41 (0)21.314.65.89 | SRTS-VD, Lausanne, director

Dr. Niels Lion | niels.lion@mavietonsang.ch | +41 (0)21.314.65.71 | SRTS-VD, Head of R&D, component production and quality control

Dr. Marie-Pierre Bousquet-Dubouch | marie-pierre.bousquet@ipbs.fr | +33 (0)5.61.17.55.44 | IPBS, Toulouse, Associate Professor

Dr. Evelyne Deery | e.deery@kent.ac.uk | +44 (0)1227.824692 | University of Kent, Canterbury, Scientist

ARTICLES

[12] **Storage-induced inhibition of the stored red blood cells multicatalytic proteinase complex proteasome is due to the accumulation of oxidized proteins** (in preparation)
Julien Delobel, Michel Prudent, Élise Gourri, Corinne Benay, Jean-Daniel Tissot, Niels Lion

[11] **Cysteine redox proteomics of the hemoglobin-depleted soluble fraction of stored red blood cells** (in preparation)
Julien Delobel, Michel Prudent, David Crettaz, Zeinab El Hajj, Beat Riederer, Jean-Daniel Tissot, Niels Lion

[10] **Anchoring of flotillin-2 to band 3 complexes during in vitro storage of erythrocytes** (in preparation)
Michel Prudent, Julien Delobel, Corinne Benay, Jean-Daniel Tissot, Niels Lion

[9] **Large scale inkjet-printing of carbon nanotubes electrodes for antioxidant assays in blood bags**
Andreas Lesch, Fernando Cortés-Salazar, Michel Prudent, Julien Delobel, Shokoufeh Rastgar, Niels Lion, Jean-Daniel Tissot, Philippe Tacchini, Hubert H Girault
Journal of electroanalytical chemistry 717-718 (2014) 61-68
DOI: 10.1016/j.jelechem.2013.12.027
Impact Factor: 2.672

[8] **Blood microvesicles: From proteomics to physiology**
Jean-Daniel Tissot, Giorgia Canellini, Olivier Rubin, Anne Angelillo-Scherrer, Julien Delobel, Michel Prudent, Niels Lion
Translational Proteomics 1 (2013) 38-52
DOI: 10.1016/j.trprot.2013.04.004
Impact Factor: to be determined (new journal)

[7] **Subcellular distribution and dynamics of active proteasome complexes unraveled by a workflow combining in vivo complex cross-linking and quantitative proteomics**
Bertrand Fabre, Thomas Lambour, Julien Delobel, François Amalric, Bernard Monsarrat, Odile Burley-Schiltz, Marie-Pierre Bousquet-Dubouch
Molecular & Cellular Proteomics 12 (2013) 687-699
DOI: 10.1074/mcp.M112.023317
Impact Factor: 7.251

[6] **Red blood cell-derived microparticles isolated from blood units initiate and propagate thrombin generation**
Olivier Rubin, Julien Delobel, Michel Prudent, Niels Lion, Kid Kohl, Erik I Tucker, Jean-Daniel Tissot, Anne Angelillo-Scherrer
Transfusion 53 (2012) 1744-1754
DOI: 10.1111/trf.12008
Impact Factor: 3.526

[5] **Red blood cell microparticles: clinical relevance**
Olivier Rubin, Giorgia Canellini, Julien Delobel, Niels Lion, Jean-Daniel Tissot
Transfusion Medicine and Hemotherapy 39 (2012) 342-347
DOI: 10.1159/000342228
Impact Factor: 1.590

[4] **Proteomic analysis of Intercept-treated platelets**
Michel Prudent, David Crettaz, Julien Delobel, Jean-Daniel Tissot, Niels Lion
Journal of Proteomics 76 (2012) 316-328
DOI: 10.1016/j.jprot.2012.07.008
Impact Factor: 5.074

[3] **Subcellular fractionation of stored red blood cells reveals a compartment-based protein carbonylation evolution**
Julien Delobel, Michel Prudent, Olivier Rubin, David Crettaz, Jean-Daniel Tissot, Niels Lion
Journal of Proteomics 76 (2012) 181-193
DOI: 10.1016/j.jprot.2012.05.004
Impact Factor: 5.074

[2] **Red blood cell microparticles and blood group antigens: an analysis by flow cytometry**
Giorgia Canellini, Olivier Rubin, Julien Delobel, David Crettaz, Niels Lion, Jean-Daniel Tissot
Blood Transfusion 10 (2012) s39-45
DOI: 10.2450/2012.007S
Impact Factor: 1.858

[1] **Biomarker Analysis of Stored Blood Products: Emphasis on Pre-Analytical Issues***
Julien Delobel, Olivier Rubin, Michel Prudent, David Crettaz, Jean-Daniel Tissot, Niels Lion
International Journal of Molecular Sciences 11(2010) 4601-4617
DOI: 10.3390/ijms11114601
Impact Factor: 2.464

** this review article was selected for presentation and discussion in a webinar organized by the America's Blood Centers SMT Journal Club (April 8th 2011)*

ORAL PRESENTATIONS

[2]Protein carbonylation in subcellular fractions of stored red blood cells: an oxidative pathway leading to microparticulation? – SPS PhD Students' Symposium 2012 (December 5th-6th, 2012, Basel, Switzerland)

[1]Oxidative stress in stored red blood cells: subcellular quantitation and identification of carbonylated proteins – SwissTransfusion 2012 (September 6th-7th, 2012, Basel, Switzerland)
Abstract published in *Clinical Laboratory* 2012; 58(7-8):S1-S7
Impact Factor: 1.056

POSTERS

[4]Red blood cell storage: an oxidative pathway model driven by protein oxidation and proteasome inhibition – SMAP 2014 (June 30th – July 2nd, 2014, Lyon, **France**)

[3]Quantitation and identification of carbonylated proteins in subcellular fractions of stored red blood cells – AABB Annual Meeting & CTTXPO 2012 (October 6th-9th, 2012, Boston, United States)

[2]Protein carbonylation during storage of erythrocyte concentrates: an accumulative event regulated by microparticulation - 10th HUPO World Congress (September 4th - 7th, 2011, Geneva, **Switzerland**)

[1]Aging and storage of erythrocyte concentrates - Swiss Proteomic Society PhD Student Symposium 2010 (December 01st-02nd, 2010, Basel, Switzerland)