Red blood cell storage time and transfusion: current practice, concerns and future perspectives

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Abstract

Red blood cells (RBCs) units are the most requested transfusion product worldwide. Indications for transfusion include symptomatic anaemia, acute sickle cell crisis, and acute blood loss of more than 30% of the blood volume, with the aim of restoring tissue oxygen delivery. However, stored RBCs from donors are not a qualitative equal product, and, in many ways, this is a matter of concern in the transfusion practice. Besides donor-to-donor variation, the storage time influences the RBC unit at the qualitative level, as RBCs age in the storage bag and are exposed to the so-called storage lesion. Several studies have shown that the storage lesion leads to post-transfusion enhanced clearance, plasma transferrin saturation, nitric oxide scavenging and/or immunomodulation with potential unwanted transfusion-related clinical outcomes, such as acute lung injury or higher mortality rate. While, to date, several studies have claimed the risk or deleterious effects of "old" *vs* "young" RBC transfusion regimes, it is still a matter of debate, and consideration should be taken of the clinical context. Transfusion-dependent patients may benefit from transfusion with "young" RBC units, as it assures longer inter-transfusion periods, while transfusion with "old" RBC units is not itself harmful. Unbiased Omics approaches are being applied to the characterisation of RBC through storage, to better understand the (patho)physiological role of microparticles (MPs) that are found naturally, and also on stored RBC units. Perhaps RBC storage time is not an accurate surrogate for RBC quality and there is a need to establish which parameters do indeed reflect optimal efficacy and safety. A better Omics characterisation of components of "young" and "old" RBC units, including MPs, donor and recipient, might lead to the development of new therapies, including the use of engineered RBCs or MPs as cell-based drug delivering tools, or cost-effective personalised transfusion strategies.

Keywords: RBCs, transfusion, storage, microparticles, Omics.

Red blood cells: description and physiological ageing

Red blood cells (RBCs), or erythrocytes, are the cells in charge of delivering oxygen to the body tissues, and carbon dioxide from the body tissues towards the lungs. They lack a nucleus and most organelles, and contain mainly haemoglobin, the specialised metalloprotein tetramere whose haeme groups actively bind gas molecules. In the circulation, an erythrocyte, which is 6-8 microns in diameter, must elongate and deform to pass through the capillaries and the splenic red pulp endothelial slits. Thus, during its 120-day life-span, the erythrocyte undergoes extensive passive deformation and must be mechanically stable to resist fragmentation[1].

Red blood cells are cleared from the circulation by two different mechanisms: RBC ageing or senescence is characterised by decreased RBC deformability and increased sphericity due to progressive surface area (membrane) loss and a decline in metabolism. Oxidative challenges to RBCs, like ageing-dependent oxidation of membrane protein Band3, increase the affinity of normally circulating anti-Band3 antibodies[2,3]. Such antibodies, when membrane bound, lead to partial complement activation on the RBC surface, which is associated with decreased RBC deformability. These immunocomplexes, in turn, are recognised by complement receptors on macrophages, which actively clear RBCs from the circulation[2,4]. The other RBC clearing mechanism is related to an ageing-independent process, which induces destruction of intact RBCs, and/or RBC vesiculation, as it occurs during inflammation[4,5].

Red blood cell transfusion: demands, guidelines and recommendations

Anaemia can be rigorously defined as a reduction in the absolute number of circulating RBCs or in one or more of the major RBC measurements obtained as part of the complete blood count (CBC), i.e. haemoglobin concentration (HGB) or haematocrit (HCT). The main consequence of anaemia is that not enough oxygen is being transported to body tissues. Depending on the

severity and duration of the anaemia, it might lead to asthenia, dyspnoea, headaches, arrythmia and/or chest pain. Depending on the cause of the anaemia and the clinical context, transfusion of RBC might be advised as a treatment. Seemingly, the transfusion needed might range from a single RBC unit to several through a short period, or to a sustained transfusion regime during a prolonged period or a life-time.

Red blood cells are the most frequently transfused blood product with approximately 85 million RBC units being transfused worldwide per year, of which 12-16 million units are transfused only in the United States[6,7]. Transfusion of RBCs should be based on the patient's clinical condition. Indications for transfusion include symptomatic anaemia, acute sickle cell crisis, and acute blood loss of more than 30% of the blood volume[7].

Transfusion guidelines have evolved through the years. The 10/30 rule, i.e. to transfuse when a patient has HGB levels of 10 g/dL or less and HCT of 30% or less, was used until the 1980s[8]. In 1999, a randomised, multicentre, controlled clinical trial evaluated a restrictive transfusion trigger (HGB 7-9 g/dL) *vs* a liberal transfusion trigger (HGB 10-12 g/dL) in patients who were critically ill. Restrictive transfusion practices resulted in a 54% relative decrease in the number of units transfused and a reduction in the 30-day mortality rate[7,9].

An optimal transfusion strategy should be considered individually and it should involve administering enough RBCs to maximise clinical outcomes while avoiding unnecessary transfusions that increase costs and expose patients to potential transfusion-related risks. Clinical context is critical in the decision to transfuse RBCs above or below the specified haemoglobin threshold[10,11]. For example, the restrictive transfusion strategy is useful for children who are stable patients in intensive care[12]. However, it should not be used in pre-term neonates or in children with severe hypoxaemia, active blood loss, haemodynamic instability, or cyanotic heart disease[7,11]. Seemingly, patients with coronary artery disease are an important subgroup that may need to be treated differently, as oxygen delivery from RBCs to the heart is critical and may be reduced by obstructed coronary arteries or anaemia[10,13].

How are conventional RBC units prepared and stored?

Red blood cell units are prepared from whole blood by removing the plasma fraction after centrifugation. One RBC unit should increase HGB by 1 g/dL and the HCT by 3%. Nowadays, RBC units are leuco-filtered before storage, which limits transfusion-related alloimmunisation, and are considered cytomegalovirus safe[7].

Preservative solutions are added to RBCs to improve their shelf-life and quality. In the early 1940s, the development of the first effective anticoagulant-preservative solution, acid citrate dextrose (ACD), allowed RBCs to be stored for up to 21 days. During the following years, an effort was made to develop other solutions, such as citrate phosphate dextrose (CPD), that allowed storage for 21 days, CPD-adenine, which allowed storage for 35 days, and the current generation of additive solutions that allow the conservation of RBCs up to 42 days at 1-6 ^{o}C[14,15]. In most European blood banks, preservatives used in RBC storage contain SAG-Manitol (saline-adenine-dextrose-mannitol)[16]. The most common are AS-1 (Adsol®; Fenwal, Lake Zurich, IL, USA), AS-3 (Nutricel®, without mannitol; Haemonetics Corporation, Braintree, MA, USA) and AS-5 (Optisol®; Terumo Corporation, Elkton, MD, USA). Nowadays, hermetic and sterile storage systems have been developed for the separation and collection of blood components in a sterile environment, even adapted for the preparation of transfusion units for paediatric patients[17].

Effect of storage conditions on the quality of the RBC units

The storage lesion is a denomination that generally includes all changes that occur as RBCs age while in storage solution. These changes result in haemolysis (with concomitant increase in extracellular free-iron, haeme and haemoglobin, resulting in nitric oxide [NO] reduced bioactivity due to scavenging), morphological changes, accumulation of lactic acid and potassium/calcium, a decrease in 2,3-DPG and ATP, decrease in pH and glycolysis rate, and an accumulation of shed bioactive proteins, lipids, and RBC-derived microparticles or microvesicles (MPs/MVs)[4,18-20]. This has led to the classification of "young" (<14-21 days) *vs* "old" (>21 days) RBC units. A recent study has put forward the significant effect of storage time and manufacturing procedure on the increase of cell-free mitochondrial DNA (mtDNA) and extracellular vesicles (EVs) in RBC units. Although the clinical relevance of these findings is unknown, both mtDNA and EVs could impact transfusion safety, as they represent damage-associated molecular patterns (DAMPs) which may potentially prime the recipient's immune system[21,22].

Accumulation of bioactive lipids during RBC storage has been identified as a potential source of post-transfusion sequelae in vulnerable populations. A targeted metabolomics study aimed at quantifying a panel of bioactive lipids in both leucoreduced (LR) and non-leucoreduced (NLR) RBC units over the course of storage showed that leucoreduction greatly attenuated the production of bioactive lipids. However, despite leucoreduction, major polyunsaturated fatty acids (PUFAs) and their oxidation products (oxylipins) were observed in the RBC units stored for 42 days[23].

"Young" *vs* "old" RBC unit transfusion: clinical aspects

The storage lesion represents a risk to efficient RBC perfusion and tissue oxygen delivery, which is the opposite of the intended effect of the RBC transfusion practice[18,24]. Some of the concerns are the potential faster clearance of "old" transfused RBC, toxicity/inflammation induced by haemolysis and free-iron, and other adverse events, including a higher infection risk or higher transfusion-dependent mortality rate[25-28]. Evidence suggests that the storage lesion affects RBC quality (haemolysis, membrane rigidity), which directly influences gas transport capacity and RBC clearance, and induces the appearance of cell-free DAMPs with consequences unfavourable to healthy homeostasis, including priming the innate immune response[25,29]. Free-iron causes plasma transferrin saturation, free haeme can cause kidney or liver damage or cardiac events, and EVs may induce recipient neutrophil priming and production of reactive oxygen species (ROS)[30,31]. These changes may produce depletion of available NO, with subsequent endothelial dysfunction predisposing to morbidity and mortality in the transfusion recipient[32]. However, other studies suggest that (overall) healthy recipients are unlikely to have baseline vascular dysfunction or a metabolic state that would easily be perturbed by transfusion-associated factors[33]. Interestingly, recent studies show that neither enhanced clearance, nor enhanced transferrin saturation, nor increased risk of transfusion-related acute lung injury (TRALI) is encountered when transfusing "old" *vs* "young" RBCs in a human endotoxaemia model[34-36].

Despite the efforts made to assess whether "old" *vs* "young" RBC should be used on a transfusion regime, this is still a matter of debate, simply because there has been no consensus on the study cohort or in evaluating the clinical consequences and parameters to be measured in such studies, and conflicting results have been presented so far[18]. It is also important to acknowledge donor-to-donor variation (due to genetic factors, sex, and social habits like smoking and diet) and the possibility that prolonged storage exposes RBCs to non-physiological stress conditions, influence storage media/container, and may amplify the effects of subclinical pathological manifestations[37]. It is worth mentioning that since RBC leucoreduction has been introduced as standard procedure in the preparation of RBC units, an increased risk of cytokine burst and alloimmunisation is not a justification to use "young" RBCs[38]. However, nowadays there is a general consensus to apply a transfusion strategy of "young" RBCs on those patients that require frequent transfusions, simply because they reduce the number of RBC units needed, thus decreasing general transfusion-related risks[25-28,39].

Two recent trials enrolled 4,000 participants across a variety of populations (cardiac surgery, critically ill, paediatric and acute hospitalised inpatients). The results of all these trials have found no clinical benefit in using fresher RBCs when compared with older or standard-issue RBCs[40]. In contrast, INFORM, a large randomised trial comparing the effect of younger *vs* older RBC units in the transfusion practice on in-hospital mortality in hospitalised patients in centres in Australia, Canada, Israel, and the USA, concludes that transfusion with "young" RBCs is associated with superior outcomes compared with standard issue RBC units, and underline the fact that consideration should be given to shortening blood storage times[41]. While it seems that the introduction of "old" RBC units does not present a specific risk, RBC storage time should be of concern in specific cases.

Anaemia

In patients with chronic anaemia secondary to congenital or acquired haematologic diseases like sickle cell disease (SCD), thalassaemia, myelodysplasia or aplastic anaemia, it is acknowledged that the transfusion of "young" (fresh or cryopreserved) RBC is more profitable, mostly because the period until the next transfusion is usually more prolonged[42,43]. In fact, as recently described, SCD trait enhances post-transfusion clearance of "old" RBC in mice, and transfusion with "old" RBC would prove very inefficient in this subjacent pathology[37]. This is easy to understand if we consider that the average life-span of transfused RBCs is about 50-60 days and can be significantly shorter in the presence of factors reducing their survival[44].

There is a consensus amongst institutions to standardise the norm of transfusion in these patients. However, there are very few institutions that have clear protocols. Most institutions suggest transfusion with less than 15-day RBC units, although policies, practices and opinions about the risks of older units for chronic anaemia patients vary a lot[45].

Critically ill, cardiac, surgery and trauma patients

Several studies have reported effects on various cardiac-related parameters due to transfusion with "old" RBCs in patients with cardiovascular disease or undergoing cardiac intervention, such as risk of in-hospital mortality, impaired pulmonary or cardiac function, and vascular disease[46]. In contrast, other studies show that there is no significant increase in adverse events including mortality in cardiac surgery patients (paediatric as well as adult) receiving "old" or "young" RBC units[47,48].

An association between storage time of RBC and length of the intensive care unit (ICU) stay was reported by Hebert *et al*. in 1999. However, this study used NLR RBCs[9,49]. More recent studies describe the development of pneumonia (but not other types of infections) that correlated significantly when receiving RBCs that had

been stored more than 28 days[29,46]. The hypothesis is that "old" RBCs enhance susceptibility to lung inflammation and induce necroptosis of the lung that may account for the increased risk of pulmonary malfunction in critically ill transfused patients; however, the mechanism is unknown[50]. Patients with septic shock benefit from a restrictive transfusion strategy but no difference has been found when comparing "young" and "old" RBCs[51]. This finding is substantiated by recent studies in a human endotoxaemia model[34-36].

In patients undergoing major gastrointestinal surgery, the incidence of post-operative complications was higher among patients who received "old" *vs* "young" blood[52]. Mynster *et al.* reported a small beneficial effect of "old" RBC in transfused colorectal surgery patients, measured as reduced cancer recurrence; however, association to transfusion-dependent immunomodulation is not proven[28]. Contradicting results regarding the use of "old" *vs* "young" RBC have also been reported for transfusion practice in brain injury patients[53,54].

Pre-term, neonate and maternity

As to the group of pre-term and neonate patients, studies show that the transfusion of "old" *vs* "young" RBCs does not have clinical consequences[55]. However, a prospective observational study of critically ill children, observed that transfusion of RBCs stored for longer than four weeks significantly increased plasma-free haemoglobin, serum iron and non-transferrin-bound iron[56]. Not many studies have examined the clinical implications of transfusion of "young" *vs* "old" RBCs in neonates and to date no significant evidence has been found[57]. In the maternity patients requiring RBC transfusion, the evidence tells us that there are no increased rates of adverse outcomes after transfusion with stored RBCs[58].

Overall, results so far lack statistical significance and clinical value, since there were multiple medical and technical limitations at the time studies were performed. Whether parameters are associated or whether they are cause or consequence is also debatable. Until reliable evidence is available, the use of "young" rather than "old" blood cannot be recommended for cardiac, ICU, surgery, trauma or paediatric patients, therefore it can be concluded that both approaches (fresh *vs* standard age blood) are safe and effective[59].

Microparticles

Microparticles or microvesicles are small phospholipid-coated particles released from cellular plasma membranes due to local cytoskeletal rearrangements and membrane budding caused by activation or apoptosis. They contain membrane proteins and cytoplasmic cargo from the mother cell, and are found in almost every body fluid[60,61]. MPs vary in size, ranging from 50 nm to 1 μm, and have a low refractive index.

During the last years, considerable progress has been made on the characterisation of blood-cell-derived vesicles and their potential roles in health and disease. The first report of the existence of MPs in blood was published almost 70 years ago (platelet "dust")[61]. Later on, MPs derived from RBCs, monocytes, endothelial cells and granulocytes, in addition to those derived from platelets, were described in plasma[60].

Genesis of microparticles

Microparticle generation results from a change in the asymmetry of the lipid bilayer of the cellular membrane. Physiological MP generation takes place concomitant to apoptosis of different cells, and the presence of MPs in the blood of healthy individuals is seemingly constant[62]. MP generation may also arise due to physiological/pathological events resulting from cell activation by agonists[63].

There are several factors that trigger MP formation during RBC storage, which include shear stress (due to the close contact between RBCs in the storage bag), anticoagulant-dependent effects, oxidative stress, calcium index and pro-apoptotic stimulation[62].

It is also known that the storage length is decisive to MP genesis. In addition, donor age and sex have been described to influence MP release upon storage, i.e. blood from female or older donors are more prone to MP formation[64].

Clinical impact of physiological and RBC unit-derived MPs

The main characteristics of erythrocyte-derived MPs (EMPs) are similar to those of MPs derived from other cells, though they are more homogeneous in size (around 0.15 μm). EMPs represent about 4-8% of the total MPs in plasma[65]. MPs derived from RBCs are linked to the occurrence of several pathologies[65-68]. In SCD, the polymerisation of abnormal haemoglobin S affects RBC membrane stability, leading to erythrocyte sickle shape and vesiculation, which correlates with the rate of intravascular haemolysis as well as the degree of coagulation activation, as EMPs are procoagulant[62]. In paroxysomal nocturnal haemoglobinuria, due to complement activation, there is an increase of EMPs from glycosyl phosphatidyl inositol-deficiency that leads, as anticipated, to a mayor incidence of procoagulant activity. EMPs are increased in patients who develop acute graft-*vs*-host disease after stem cell transplantation, although the mechanism is not clear. Elevated levels of EMPs have also been observed in cases of malaria infection, especially with *Plasmodium falciparum.*

In the context of transfusion medicine, MPs in the RBC unit are considered a sign of the RBC storage lesion, and thus may be involved in some effects described in patients transfused with blood stored for more than 21 days when RBC membrane stability is

lost and haemolysis takes place in the storage bag[66,67]. It is well established that stored RBC concentrates have been associated with pathological reactions ranging from immunomodulation and pro-inflammation post-transfusion[22]. EMPs act on the innate immune system as paracrine messengers and as pro-inflammatory mediators inducing or propagating inflammatory signals[69].

MPs derived from stored RBCs are linked to the occurrence of the following transfusion-dependent adverse events. TRALI is among the most serious of the transfusion-related adverse events with high morbidity and mortality. A recent study postulates that EMPs are likely to be mediators for TRALI, by the following evidence. First, it has been shown that EMPs can bind and activate neutrophils. Second, complement and IgG are enriched in EMPs from "old" RBC due to the storage lesion. The EMP-bound IgG and complement can activate neutrophils *via* Fc receptors. These two events lead to the development of TRALI. In addition, EMPs exhibit potent pro-coagulant activity so they may contribute to post-transfusion thrombosis. This could be explained by the fact that EMPs express the anionic phospholipid phosphatidylserine (PS), which serves for assembly of the coagulation factors into active complexes for thrombin generation[65-68].

It is known that blood sample processing is likely to trigger artificial MP formation during collection, transportation, and storage. Though careful blood sample handling is crucial, some other precautions should be taken into account: 1) discarding the first few millilitres of blood collected, as they are more prone to activation; 2) choosing the proper anticoagulant (citrate); 3) transporting blood samples horizontally; 4) performing the first centrifugation at 1,500 *g* within two hours of blood collection, followed by a second centrifugation at 13,000 *g*[62].

The identification of MPs on a sample, plasma or an RBC concentrate transfusion unit is mainly performed by flow cytometry[70,71]. The combination of the right FSC and SSC, and threshold settings in the cytometer, with RBC membrane surface markers such as CD235a and CD47, is crucial to distinguish EMPs from other MPs[71]. Application of other dies such as carboxy fluorescein diacetate succinimidyl ester (CFSE) in combination with Glycophorin/Annexin V allows the cellular origin of these elements to be determined or followed up[70].

Nowadays, MPs are a great "hotspot". They are studied as: 1) biomarkers for diagnosis and prognosis; 2) monitoring therapeutic efficacy and patient risk stratification, as well as, personalised medicine; and of course, 3) various emerging therapeutic applications. However, in transfusion medicine, they are considered a risk to avoid. Since MPs occur with higher frequency at longer storage periods, the difference between "old" and "young" RBC should be made when clinically relevant to avoid MPs in the transfused product[68].

However, there are still some questions that remain unanswered. Are all EMPs equal in a qualitative manner? Are EMPs generated in a storage bag the same as physiological EMPs in health and in disease? Can we extrapolate the effects of RBC unit EMPs from pathophysiological EMPs? While some associations have been made related to the harmful or safety hazards of EMPs, both in pathological conditions where their production is enhanced as well as transfusion-dependent, there is no study to date that can conclude whether EMPs are harmful at all or, if so, in which way. For gaining insight into this matter, proteomics approaches are being applied to characterise and define EMPs properties at the quantitative and qualitative levels[71]. This leads us to our next question: could EMPs have any benefit at all, or could we learn from their physiology to apply them (in a controlled manner) as treatment for certain pathologies[62]? And how do transfused RBC interact with recipient MPs, especially in those transfusion-dependent patients who present them in a pathophysiological manner?

Strategies to keep RBC "younger"

Storage of RBCs induces progressive biochemical and biomechanical changes that affect red cell viability, deformability, oxygen carrying capacity and microcirculatory flow[72]. Despite the contradictory data reported, there is evidence from systematic analysis that supports the notion that the deleterious effect of RBC storage has not only a direct relationship to the efficacy of transfusion, but also adverse clinical consequences, especially in certain recipients[39]. Therefore, an inadequate transfusion strategy has not only a negative clinical impact, but also significant economic consequences. Efforts are being made, therefore, to improve the shelf-life, but also the quality of the RBC units, through unbiased approaches of characterisation of the RBC unit during storage. Some of the current alternatives might have a specific application and context-dependent cost-effectiveness.

Cryopreservation

Storage of RBCs at ultra-low temperature halts the cellular metabolism, preventing the progressive deterioration that is responsible for the RBC storage lesion, allowing preservation of RBC units for prolonged periods. The first successful cryopreservation of human RBCs in glycerol was reported by Smith *et al.* in 1950, and soon after this, the first transfusion of human cryopreserved RBCs was performed. By the early 1960s, several thousand units had been frozen, thawed and transfused. Nevertheless, the use of

cryopreservation has been hampered by the complex freezing process, elevated cost and the limited shelf-life of thawed products. The subsequent development of more sophisticated post-thaw washing and additive solutions have made cryopreserved RBCs better suited for clinical practice[73-75]. There are several freezing/thawing/deglycerolisation method procedures. Importantly, the deglycerolisation washing process also reduces detrimental substances, such as bioactive lipids, cytokines, potassium and free haemoglobin, and removes residual leucocytes that remained after the buffy-coat repletion. Cryopreserved washed RBCs contain hardly any biologically active substance in the supernatant.

However, in cryopreserved RBCs the natural history of the storage lesion is altered[73,74]. While ATP and 2,3-DPG levels are preserved while frozen, they decline post-thaw rapidly, and RBC agglutination profile is hampered. On the other hand, cryopreservation seems not to be detrimental to the deformability of RBCs. Nowadays, cryopreserved RBCs are primarily used in different settings where the RBC availability is limited or unpredictable: 1) military practice/battle field; 2) to store RBCs from rare blood groups; 3) to bridge over supplies during short-term RBC deficits (as occurs during natural or civil disasters); 4) for autologous RBC transfusion when RBC count recovery is required prior to surgery; 5) for patients with immunoglobulin A deficiency[73-75].

Anaerobic storage

Oxidative damage occurs as early as the first 7-14 days of RBC storage. Furthermore, gamma-irradiation and pathogen inactivation technology produces ROS that induce RBC oxidation leading to reduced RBC deformability and survival *in vivo*[76].

It has been hypothesised that anaerobic storage would significantly attenuate the oxygen-dependent damage to RBC and have advantages over the conventional (aerobic) storage. Anaerobic storage of RBC has been shown to diminish the overall rate of storage lesion development (haemolysis, vesicle production and phosphatidylserine exposure)[77]. However, perfusion of RBC *in vitro*, which decreases with storage, is independent of aerobic or anaerobic storage[11]. Interestingly, anaerobically stored RBC showed a significant reduction in the number of poorly-deformable RBC. Despite the implications this fact has on increasing the efficacy of transfusions (more oxygen delivery to the tissues), more clinically relevant studies need to be performed.

Donor selection: the G6PD paradigm

As oxidative injuries may play an important role in the evolution of the RBC storage lesion, the enrolment of patients with enzymopathies compromising their redox metabolism, such as G6PD-deficient ($G6PD^-$) subjects, has been considered. According to World Health Organization (WHO) guidelines, blood can be accepted from $G6PD^-$ individuals without a history of haemolysis; however, their blood is not suitable for intrauterine transfusion, neonatal exchange transfusion, or for patients with $G6PD^-$.

Several studies have reported that $G6PD^-$ RBCs behave similarly or better than G6PD-sufficient donors, as extrapolated from calcium, ATP and 2,3-DPG levels, morphology-related parameters, and a tendency for higher mean corpuscular haemoglobin and volume. However, experiments using an *in vitro* model of transfusion suggested that transfusion with $G6PD^-$ blood could result in increased haemolysis, mechanical fragility and ROS accumulation in certain situations when other "insults" are present, such as infection or oxidative stress-inducing medications. Moreover, higher levels of complement and coagulation cascade components in vesicles from $G6PD^-$ subjects have been detected, suggesting that units from these donors might promote inflammatory responses associated with transfusion, i.e. TRALI and hypercoagulability[78,79].

On the other hand, the chronic exposure of $G6PD^-$ subjects to oxidative stress might make them good recipients, as they better tolerate exposure to oxidative damaged long-stored healthy RBCs and supernatants[79].

Nitric oxide adjuvant treatment

During normal conditions, the interaction of blood flow with the vascular endothelium stimulates the production of endogenous NO. Upon transfusion, the presence of RBC damage or storage lesions causes disturbance in the cardiovascular homeostasis at different levels, many of which are closely related to the availability and function of NO. Restoration of NO production, availability and function could be a key point in transfusion success[80].

For example, inhaled gaseous NO prevents pulmonary and systemic hypertension induced by transfusion. Other approaches to resolve NO deregulation induced by transfusion include nitroglycerin supplementation, which transiently decreases vasoconstriction, or the engineering of NO-containing nanoparticles.

Animal model-based studies show that NO supplementation is a promising area to improve adverse events of "old" transfused RBC units[81]. Further studies are needed to examine these NO restoration strategies and translate them into improved outcomes in transfused patients[80,82].

Alternative additives and/or rejuvenation solutions

Many groups have proposed alternatives to restore redox imbalance by supplementing storage solutions with antioxidants, such as vitamin C (ascorbic acid)[83,84],

serotonin and ASs 90[85,86]. Other studies offer a novel mechanism for improving the quality of stored blood by inhibition of acid sphingomyelinase with amitriptyline, stabilising the erythrocyte membrane. Transfusion of stored RBCs treated with amitriptyline resulted in a dose-dependent reduction in acid sphingomyelinase activity, ultimately preventing lung inflammation in mice[87,88].

Alternatively, novel studies in mice have demonstrated the potential benefit of co-infusion with haemopexin or haptoglobin in the resuscitation of haemorrhagic shock with stored RBCs, increasing the survival rate and decreasing tissue inflammation. Interestingly, only co-infusion of haptoblobin with RBCs prevented haemoglobinuria and kidney injury[89].

Protein carbonylation and microparticles

Protein carbonylation results in irreversible protein oxidation lesions and is a marker of oxidative stress and ageing, altering active sites and protein structure of the affected proteins, globally named as carbonylome. Its occurrence during RBC storage is time- and cellular compartment-dependent. According to recent studies, antioxidant and chaperone proteins, members of the signalosome and proteosome complexes and several enzymes are extremely sensitive to carbonylation[90]. The vesiculation process allows the elimination of part of the carbonylated proteins from stored RBCs. Therefore, vesiculation and MPs formation might, in this case, be beneficial in order to remove excess carbonylated proteins. Could we use engineered MPs to remove excess carbonylated proteins from "old" RBC units, if they are proven to be harmful upon transfusion? Or could we use certain carbonylated targets as biomarkers for discarding an "old" RBC unit not suitable for transfusion?

Omics as a tool in Transfusion Medicine

Omics studies have been often criticised for their small contribution to the field of Transfusion Medicine due to their descriptive and observational nature. Nevertheless, recent efforts to correlate metabolomics/proteomics data and transfusion-relevant variables, such as 24-hour recovery for transfused RBCs, promise to boost the design and testing of innovative storage strategies[91]. Metabolomic technologies have developed thanks to recent advancements in mass spectrometry and the bioinformatics integration of Omics data[92].

Metabolomics analyses have been performed on RBCs from animal and *in vitro* models in order to understand the role of metabolites involved in oxidative lesions of stored RBCs, and it has been reported that 8 extracellular compounds (lactic acid, nicotinamide, 5-oxoproline, xanthine, hypoxanthine, glucose, malic acid, and adenine) are sufficient to determine the metabolic age of the RBC product[93]. Metabolomic studies have shown that specific oxidative and metabolic lesions, exacerbated by storage under hyperoxic conditions, were ameliorated by hypoxic storage[94]. However, although oxidative stress was less sustained in anaerobically stored RBCs compared to aerobically stored counterparts, oxidative stress markers still accumulate over anaerobic storage progression[95]. Of note, the appreciation of altered homeostasis in the purine catabolism pathway, leading to the generation of urate in humans and allantoin-allantoate in mice, has supported the notion of a potential role for donor-specific pre-storage urate levels in attenuating oxidative lesion during routine storage of RBCs[92]. Thus, progressive accumulation of oxidative stress markers such as oxidised lipids and purine homeostasis products, which are critical for membrane and intracellular signalling, have been documented in RBCs and supernatants in different ASs. Notably, energy and redox homeostasis related variables have been found to be better preserved in low-chloride, high-bicarbonate, or alkaline solutions such as PAGGGM, AS-3, and AS-7[16,96].

Perhaps RBC storage time is not an accurate surrogate for RBC quality and there is a need to establish which parameters do reflect optimal efficacy and safety[97]. Studies will then be needed to determine how to define RBC quality and if changes in these metabolic or biochemical profiles (cell deformability, oxidative injury, NO metabolism, free iron, MPs or OMICs approaches) are more accurate surrogates for RBC quality and if they are clinically relevant. In particular, a targeted proteomics analysis of the RBC unit supernatant revealed novel biomarkers of the RBC storage lesion and promises to become a key analytical readout for the development and testing of alternative cell processing strategies[98]. Additionally, baseline characteristics of donors, such as membrane Peroxiredoxin-2 and serum uric acid concentration, have been proposed as candidate biomarkers of storage quality[99]. RBC storage age time is still a candidate quality metric that requires further examination. It is likely that not just one parameter will reflect quality, and not only that, a more personalised strategy will have to be implemented, where the quality assessment of the RBC unit is matched to the clinical needs of the patient[24]. A recent study stratifying results based on metabolomics data, suggests that the RBC unit metabolic profile correlates with endothelial damage and clinical outcome, much better than the RBC parameters traditionally used[100]. A better Omics characterisation of "young" and "old" RBC units, including MPs, donor and recipient, might lead to the development of new therapies, including the use of engineered RBCs or MPs as cell-based drug delivering tools, or personalised transfusion strategies (Figure 1).

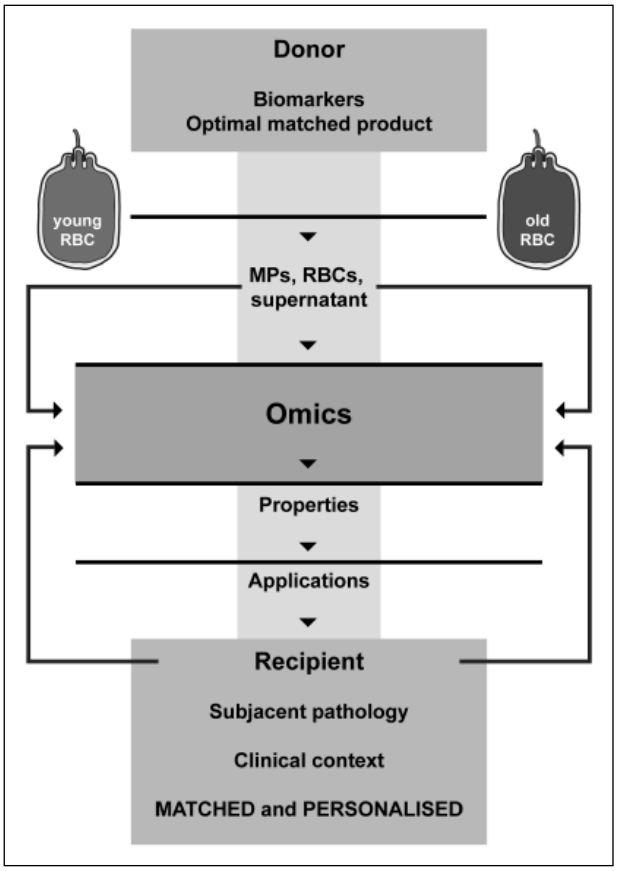

Figure 1 - Scheme depicting the future of Omics approaches for the development of personalised transfusion strategies and novel cell-based or transfusion-related therapies. MP: microparticle; RBC: red blood cell.

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