Red blood cell storage lesion: causes and potential clinical consequences

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Abstract

Red blood cells (RBCs) are a specialised organ that enabled the evolution of multicellular organisms by supplying a sufficient quantity of oxygen to cells that cannot obtain oxygen directly from ambient air via diffusion, thereby fueling oxidative phosphorylation for highly efficient energy production. RBCs have evolved to optimally serve this purpose by packing high concentrations of haemoglobin in their cytosol and shedding nuclei and other organelles. During their circulatory lifetimes in humans of approximately 120 days, RBCs are poised to transport oxygen by metabolic/redox enzymes until they accumulate damage and are promptly removed by the reticuloendothelial system. These elaborate evolutionary adaptions, however, are no longer effective when RBCs are removed from the circulation and stored hypothermically in blood banks, where they develop storage-induced damages ("storage lesions") that accumulate over the shelf life of stored RBCs. This review attempts to provide a comprehensive view of the literature on the subject of RBC storage lesions and their purported clinical consequences by incorporating the recent exponential growth in available data obtained from "omics" technologies in addition to that published in more traditional literature. To summarise this vast amount of information, the subject is organised in figures with four panels: i) root causes; ii) RBC storage lesions; iii) physiological effects; and iv) reported outcomes. The driving forces for the development of the storage lesions can be roughly classified into two root causes: i) metabolite accumulation/depletion, the target of various interventions (additive solutions) developed since the inception of blood banking; and ii) oxidative damages, which have been reported for decades but not addressed systemically until recently. Downstream physiological consequences of these storage lesions, derived mainly by *in vitro* studies, are described, and further potential links to clinical consequences are discussed. Interventions to postpone the onset and mitigate the extent of the storage lesion development are briefly reviewed. In addition, we briefly discuss the results from recent randomised controlled trials on the age of stored blood and clinical outcomes of transfusion.

Keywords: blood transfusion; blood banking; storage lesion; clinical sequelae.

Introduction

Approximately 25 trillion red blood cells (RBCs) circulate in the bloodstream of an adult individual, each one packed with ~260 million haemoglobin molecules. To make room for haemoglobin, erythroblasts and reticulocytes progressively lose nuclei and organelles during maturation, which impairs the erythrocytes' capacity to synthesise new proteins during their 120 days lifespan in the human bloodstream. Indeed, haemoglobin occupies 95% of the ~110 fL mean RBC volume[1], making up ~670 g of the 25 kg dry body weight of an average adult individual[2]. Each alpha and beta globin subunit in the haemoglobin tetramer contains one ferrous iron, which can bind one molecule of oxygen. Evolution has shaped haemoglobin and the RBC as a highly specialised carrier of oxygen in the body[3], enabling large warm-blooded vertebrates to thrive. When RBCs are fully oxygenated, concentrations of both iron and oxygen approximate 16 mM, a very high concentration considering the highly reactive nature of ferrous iron and oxygen. To mitigate oxidative stress and oxygen consumption, mature RBCs lose mitochondria and strengthen their antioxidant systems to specifically maintain haemoglobin iron in a reduced state, even in the presence of high oxygen concentrations. Oxidised (ferric state, +III) haemoglobin (i.e., methaemoglobin) can thus be either reduced back to ferrous state (+II) by enzymes such as methaemoglobin reductase, or denatured/aggregated (Heinz bodies) before removal via vesiculation (e.g., aging RBCs shed one microvesicle per hour)[4]. Senescent erythrocytes are usually characterised by higher oxidative stress than young erythrocytes and are readily removed from the bloodstream via phagocytosis by reticuloendothelial system macrophages in the liver and spleen.

In blood centers, donated blood is separated into a red cell concentrate (RCC) from which white blood cells (WBCs) are filtered in most cases, as well as platelets (log 2 WBC removal via buffy coat depletion or log 3.5-4 WBC and log 2.5 removal if leucofiltration is performed, respectively). Isolated RBCs are resuspended

in an acidic additive solution at approximately 60% haematocrit and stored under refrigerated conditions (1-6 °C hypothermic storage) for up to 3-7 weeks. In cryopreserved RBCs with a shelf life of over 10 years, chemical reactions in RBCs are virtually suspended by storing at −65 °C with a cryo-protectant. However, the latter process is time-consuming and cumbersome, and it promotes the lysis of the older, more fragile RBC population that was originally frozen[5]. In contrast, during hypothermic storage (1-6 °C), chemical reactions proceed - albeit at reduced rates[6] - without the full benefit of the protective mechanisms that operate in the circulation. Thus, gradual degradation of various components of RBCs, collectively referred to as the "storage lesion", accumulates during hypothermic storage resulting in a limited shelf life of up to 7 weeks. This contribution provides an overview of the RBC storage lesion and its potential clinical implications by examining causative elements, damage to RBCs, consequences *in vitro* and in animal models, and finally, associated clinical sequelae based on a thorough and extensive review of the existing literature.

Elements of the storage lesion and downstream consequences

Reviews[7-9] of recent randomised controlled trials (RCTs)[10-15] indicated that transfusion of the freshest available blood does not decrease the risk of mortality in several categories of recipients (including a small number of massively transfused critically ill or sickle cell disease patients) when compared to the standard of care. Despite reassuring evidence from RCTs, there is a burgeoning literature on the potential clinical sequelae other than mortality to transfusion of packed RBCs[16,17] and on the potential etiological link between the storage lesion and untoward consequences upon transfusion.

In Figure 1 we summarise elements of the RBC storage lesion - from causes to associated clinical sequelae - in four vertical panels, including root causes (Panel I); effects on RBCs (i.e., storage lesions) (Panel II); physiological consequences deduced from *in vitro* experiments or animal models (Panel III); and finally, potential clinical sequelae of RBC transfusion as gleaned from retrospective or prospective studies (Panel IV). Representative references for each of the elements in Figure 1 are provided. Our categorisations, though helpful from a systematic perspective, may at times appear arbitrary, owing to the labile boundary between cause and effect for some of the extensively reported lesions. For example, ion homeostasis is controlled by energy-dependent mechanisms, which are in turn affected by redox and energy metabolism. Nonetheless, storage temperature alone negatively affects proton pumps, and dysregulation of ion homeostasis (e.g. calcium[18]) affects kinase activity and metabolic signalling, making it difficult to conclude whether some of the proposed connections (if any) are only unidirectional. Nonetheless, we firmly believe that such a systematic overview of the storage lesion is unprecedented and will, at least, fuel further debate on the most relevant etiological factors to be targeted by next generation storage strategies/additives designed to improve RBC storage quality, as well as analytical strategies to provide pre-clinical insights regarding RBC safety and efficacy.

Root causes [Figure 2, Panel I]

For hypothermic storage, RBCs are isolated from plasma and suspended in an acidic solution containing a high concentration of glucose. During storage, RBCs are exposed to plasticisers in the storage bag as well as oxygen diffusing in from ambient air, while accumulating metabolic waste resulting in further acidification throughout the shelf life. RBCs have evolved to cope with oxidative and mechanical stresses they encounter while performing their vital function as oxygen carriers *in vivo*. However, during their isolated state under hypothermic storage *ex vivo*, RBCs face a different set of chemical and mechanical stresses. Since RBCs were never exposed to evolutionary pressure to cope with such conditions, no physiological countermeasures evolved to address the stresses that create the storage lesions. The root causes of the development of the RBC storage lesion can be roughly classified into two categories: (i) arising from isolation of RBCs, dilution of plasma with an additive solution, and extended hypothermic storage in a closed bag; and (ii) arising from storage *ex vivo* in the presence of oxygen, resulting in oxidative stress and loss of biochemical countermeasures that were functional *in vivo*. Both causes result in physical damage and biochemical impairment to stored RBCs.

Oxidative damage as a cause for RBC storage lesions [Figure 2, Panel Ia]

Chemical oxidation of iron in haemoglobin is the central reaction that initiates oxidative stress, the major element for the development of the storage lesion, in stored RBCs. RBCs contain high concentrations of reactive ferrous iron in the haeme prosthetic group of haemoglobin together with a high concentration of dissolved oxygen. Four iron moieties (ferrous state) in haemoglobin react chemically with oxygen to form methaemoglobin (ferric state). As a byproduct, superoxide anion

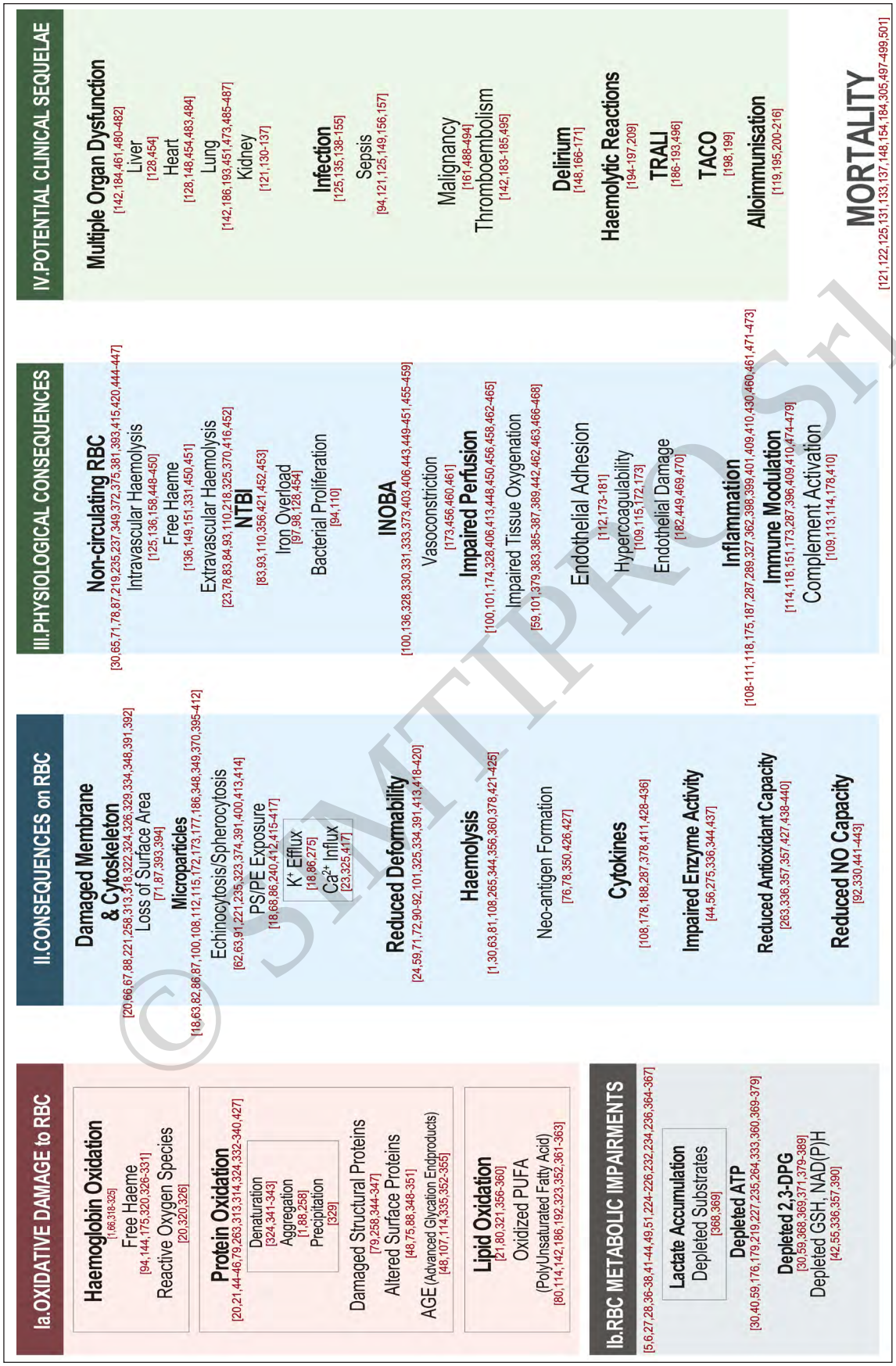

Figure 1 - Elements of red blood cell storage lesions from root causes to potential clinical sequelae.
Representative references for each element are shown within the figure. RBC: red blood cell; ATP: adenosine triphosphate (ATP); DPG: diphosphoglycerate; GSH: glutathione; NAD(P)H: nicotinamide adenine dinucleotide phosphate; PS: phosphatidylserine; PE: phosphatidylethanolamine; NTBI: non-transferrin bound iron; INOBA: insufficient nitric oxide bioavailability; TRALI: transfusion-related acute lung injury; TACO: transfusion-associated circulatory overload.

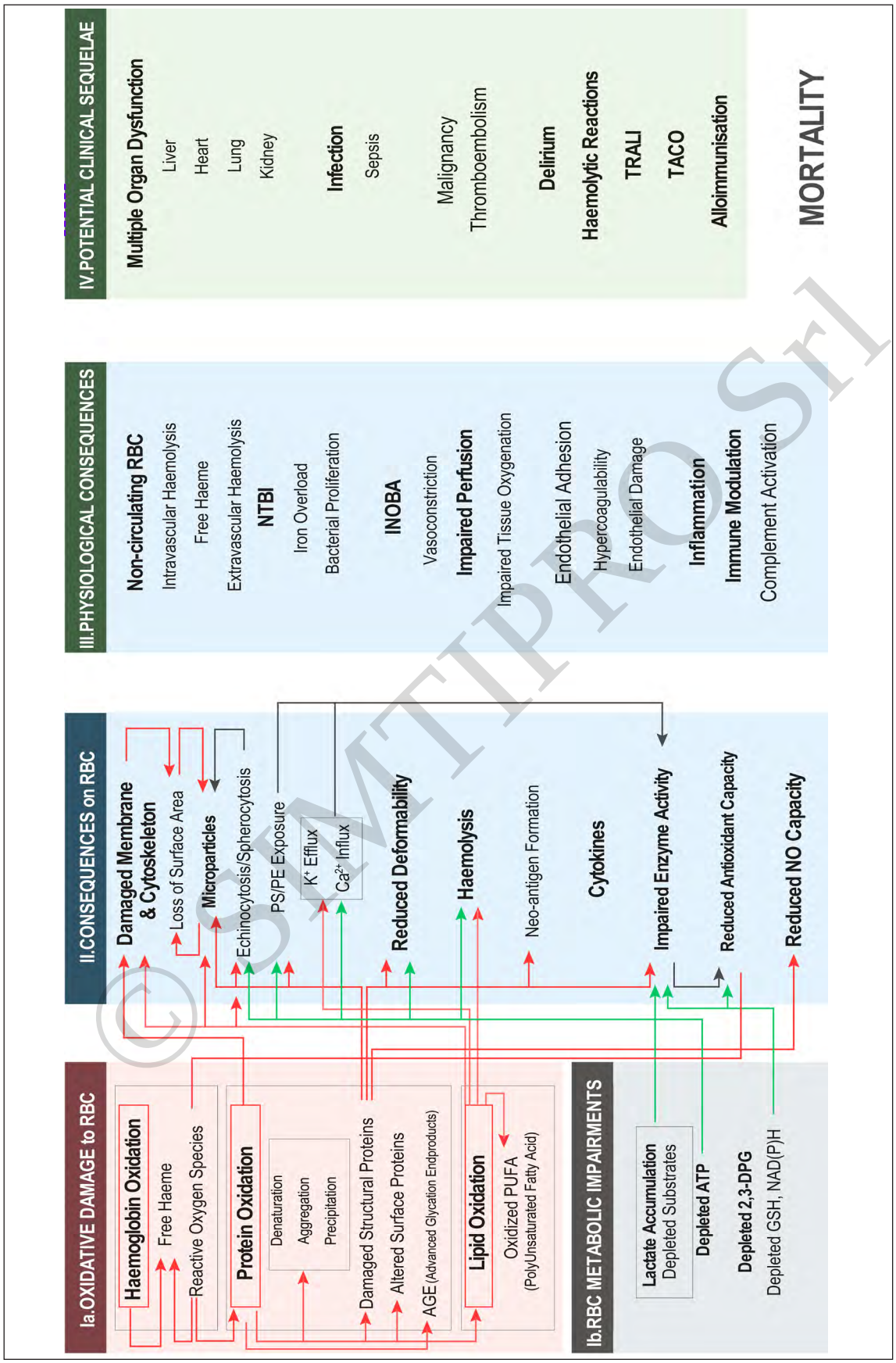

Figure 2 - Causes of red blood cell damage and impairments during storage, and effects on red blood cell as storage lesions.
RBC: red blood cell; ATP: adenosine triphosphate (ATP); DPG: diphosphoglycerate; GSH: glutathione; NAD(P)H: nicotinamide adenine dinucleotide phosphate; PS: phosphatidylserine; PE: phosphatidylethanolamine; NTBI: non-transferrin bound iron; INOBA: insufficient nitric oxide bioavailability; TRALI: transfusion-related acute lung injury; TACO: transfusion-associated circulatory overload.

is generated, which is converted by superoxide dismutase to form H_2O_2, a major reactive oxygen species (ROS) and a substrate for hydroxyl radical (OH•) generation. *In vivo*, methaemoglobin is reduced back to haemoglobin by reductase enzymes, but these enzyme activities are curtailed under hypothermic storage conditions. Coupled with higher dissolved oxygen concentrations stemming from increased solubility at low temperature, this phenomenon results in enhanced production of methaemoglobin and superoxide anion. However, methaemoglobin does not accumulate to high levels in stored RBCs, and instead denatures into globin and haemin or free haeme due to its instability at hypothermic temperature. Ferric iron in haeme is reduced by the superoxide derived from methaemoglobin formation. The resulting ferrous ion then reacts with H_2O_2 by the Fenton reaction to produce net OH• by the Haber-Weiss reaction[19]. H_2O_2 also reacts with oxyhaemoglobin to produce ferryl (+IV) haemoglobin. Both OH• and ferryl haemoglobin are highly reactive, and oxidise nearby enzymes and lipids. The occurance of this cascade of events exponentially increases after two weeks of storage in the SAGM additive, resulting in reversible and irreversible oxidations[a] of structural (e.g. ankyrin and spectrin[20]), functional (band 320 and haemoglobin[21]), and metabolic enzymes[b], exacerbating metabolic impairments[22]. These events lead to the exposure of phosphatidylserine on RBC surfaces (a phenomenon counteracted through the expenditure of high energy phosphate compounds, such as adenosine triphosphate (ATP), and imbalances in K^+ and Ca^{2+} ions[23]. Oxidised proteins, including denatured haemoglobin, bind to the cytoskeleton and disrupt its network structure causing morphological changes and reduced in deformability[24,25]. Oxidised and denatured proteins aggregate and precipitate in/on the RBC membrane. ROS also oxidises haemoglobin at the critical amino acid residue, β-92 histidine, destroying its ability to bind oxygen[21]. Another critical aspect of haemoglobin oxidation/ROS generation is lipid oxidation. Ferryl-haemoglobin and OH• are powerful ROS that can initiate the lipid peroxidation cycle[26], which is sustained by the availability of oxygen, thereby disrupting the membrane bilayer and producing biologically active, oxidised polyunsaturated fatty acids (oxylipins)[27]. Of note, in a murine model, lipid peroxidation is a predictor of post-transfusion recovery; that is, the percentage of transfused erythrocytes that still circulate at 24 h from transfusion, a minimum but not sufficient condition for transfused RBCs to exert their function[28].

Metabolic impairments as a cause for storage lesion development [Figure 2, Panel Ib]

Metabolic impairments of stored RBCs occur as a consequence of removing RBCs from a donor's circulation, isolating them from plasma, and storing them in acidic solution, with a limited solution volume, at hypothermic temperature. Depletion of critical substrates (i.e., extracellular nutrients, such as glucose and intracellular purine derivatives, such as urate[29]) and accumulation of metabolic waste products, dominated by lactic acid, occur in component processing and storage. The consequences of glycolysis are reduced pH and impaired activities of critical enzymes that supply energy and antioxidant defense[30], as reported since the 1940s[31]. Several metabolic pathways not expected in RBCs because of their lack of organelles have been discovered during the last two decades, especially with the advancement provided by "omics" sciences[32-35]. Down- and up-regulated pathways were quantified in MAP and phosphate-adenine-glucose-guanosine-gluconate-mannitol (PAGGGM) additive solutions with differences between the 0-7 day and 8-35 day periods[36,37], and a three-phase temporal evolution in metabolic pathways was reported in saline-adenine-glucose-mannitol (SAGM) and AS-3[6,38-43].

Low pH additive solutions (5.5-6.0) reduce the activities of the rate-limiting enzymes of glycolysis, the pentose phosphate pathway (PPP), and the Rapoport-Luebering shunt[c], and contribute to the rapid depletion of 2,3-diphosphoglycerate (2,3-DPG) and a gradual reduction of ATP during storage. Glycolytic enzymes are reversibly and irreversibly oxidised progressively during storage[44-46], and 2,3-DPG breakdown fuels ATP generation at the expense of haemoglobin capacity to off-load oxygen, due to the ensuing low 2,3-DPG levels. As 2,3-DPG is consumed and haemoglobin oxygen saturation is increased by storage weeks 2-3, ROS accumulation reaches its plateau in classic additives, such as SAGM[20].

It is worth noting that glucose availability is not a limiting factor, since all of the currently available additives are loaded with glucose to such an extent (>50 mM and up to 154 mM in the case of AS-1) that glucose autoxidation and non-enzymatic glycation of haemoglobin (HbA1c[47]) and membrane proteins[48] are observed in end-of-storage RBCs.

[a]For example, beta-elimination of cysteine thiols to generate dehydroalanine or carbonylation on side chains of lysine, arginine, proline and threonine residues[20,44,45,314-316].

[b]For example, glyceraldehyde 3-phosphate dehydrogenase[44] and peroxiredoxin[286,314,317].

[c]For example, phosphofructokinase, glucose 6-phosphate dehydrogenase and biphosphoglycerate mutase.

Depletion of ATP and nicotinamide adenine dinucleotide phosphate $(NADP)^+$ becomes a limiting factor in glutathione synthesis (an ATP-dependent process), resulting in reduced glutathione pools during storage. As the γ-glutamyl cycle is incomplete in mature RBCs, the lack of oxoprolinase activity results in 5-oxoproline accumulation in stored RBCs and supernatants, representing a key marker of RBC metabolic age during storage[42]. These considerations explain why attempts to replenish glutathione reservoirs by feeding RBCs in additives supplemented with glutathione precursors (e.g. glutamine) have failed to date[49,50].

Of note, redox and energy metabolism in stored RBCs are more intertwined than was generally assumed for decades. Recent evidence suggests that hypoxanthine accumulates in stored RBCs as a result of ATP breakdown into adenosine monophosphate (AMP), whose deamination to the hypoxanthine-precursor inosine monophosphate (IMP) is enzymatically catalysed by RBC-specific AMP deaminase 3 in response to oxidative stress[51]. This is relevant in that hypoxanthine is a predictor of post-transfusion recovery in mice and, preliminary data suggest, in humans[51]. At the end of storage, hypoxanthine in RBCs and supernatants are at mM levels; hypoxanthine at ~100 μM is a substrate for generating hydrogen peroxide and urate in the presence of xanthine oxidase in the circulation of transfusion recipients. This prompts the consideration that circulating levels of post-transfusion hypoxanthine above the 100 μM threshold (i.e., the equivalent of a single end of storage ~450 mL unit diluted in 5 L of blood in the recipient) may be sufficient to catalyse oxidative stress, negatively impacting the recipient including the recipient's RBCs[52].

Some of the metabolic lesions that RBCs undergo during refrigerated storage are somewhat reversible following transfusion. For example, ATP and 2,3-DPG levels recover by 7-72 h after circulating in the recipient[53], though at a rate that may be insufficient to meet the sudden and supra-physiological metabolic demand of trauma or critically ill recipients. Furthermore, RBCs that were near senescent when collected for storage, with reduced ATP and enzyme activities, may not be able to recover from the metabolic impairment and be removed by the recipient's reticuloendothelial system after transfusion, as studies on the effect of the storage lesion on RBC populations sorted through density gradients seem to suggest[54].

Consequences for RBCs - storage lesions [Figure 2, Panel II]

Extensive metabolomic investigations revealed that levels of high-energy compounds, such as ATP and 2,3-DPG, as well as reducing equivalents, glutathione (GSH) and (NAD(P)H), are reduced with a shift in the overall metabolic state after approximately two weeks of hypothermic storage[55]. Depleted ATP impacts several enzymatic functions and ion pumps, such as Ca^{2+} pumps. Decreased ATP deregulates cation homeostasis and disrupts membrane asymmetry, triggering the exposure of phosphatidylserine (PS) and phosphatidylethanolamine (PE), normally confined to the inner bilayer, and leading to microparticle formation. ATP depletion also alters the ability of kinases to phosphorylate proteins, as revealed by the restored membrane protein phosphorylation capacity after rejuvenation of long-stored RBCs[56]. Depleted ATP also causes reorganisation of the cytoskeleton, leading to echinocytosis[57]. Depletion of reducing equivalents results in reduced anti-oxidant capacity, further exacerbating damage from oxidative stress during storage and in RBC recipients after transfusion. Rapid loss of S-nitrosylation (SNO) of haemoglobin in stored RBCs is hypothesised to interfere with vasodilation in transfused patients[58] by causing insufficient nitric oxide bioavailability (INOBA), although SNO-haemoglobin's relevance remains controversial[59,60].

A unit of RBC contains a continuum of cell ages from those just released into the circulation to those that are senescent and at the end of their circulatory life. Most *in vitro* parameters described in this panel are averaged values from this inhomogeneous RBC population in which the rate of damage accumulation may not be linear with storage time nor consistent from donor to donor. Thus, a unit of stored RBCs at any storage time contains some senescent cells that have lost excess membrane area by vesiculation and have decreased antioxidant capacity (e.g. glucose 6-phosphate dehydrogenase activity decreases in older circulating RBCs[61]). Morphological analyses report 6-9% of RBCs with irreversible changes[62,63]. Additionally, those cells' metabolic status may have exhausted their capacity to handle oxidative stress during *ex vivo* storage for an extended time[54], explaining the average ~17.6% loss of potency of end of storage packed RBCs when transfused back to a healthy autologous donor[64] as gleaned from extensive post-transfusion recovery studies[65]. Reorganisation and damage to the RBC membrane and cytoskeleton, binding of haemoglobin and oxidised proteins, degradation of band 3, and variations in raft proteins[66,67] are consequences of hypothermic storage. Accumulation of denatured methaemoglobin and damage caused by ROS result in changing RBC morphology from discocytes to echinocytes, then irreversibly to spherocytes, by releasing microparticles (MPs), resulting in reduced deformability that is not reversible after transfusion. Oxidation of membrane lipids and proteins exposes inner membrane phospholipids (PS and PE)[68-70],

contributing to membrane re-organisation and promoting MP formation. Proportionally higher loss of membrane area compared to volume occurs with MP formation, leading to loss of the excess surface area needed to allow passage of RBCs through narrow splenic capillaries[71]. Minimisation of surface to volume ratios also increases RBC osmotic fragility[62]. Cross-linking cytoskeleton and membrane proteins[72], dysregulating cytoskeletal protein phosphorylation[73], and dehydration caused by Ca^{2+} influx and K^{+} efflux, also contribute to reduced RBC deformability[74]. Oxidation of the membrane cytoskeleton disturbs anchoring between membrane proteins and the cytoskeleton, leading to eryptosis signal formation by band 3 clustering[25]. Oxidation of CD47 leads to eryptosis signal formation75, while storage-dependent depletion of membrane CD47[76,77] - a "do not eat me" signal - makes transfused RBCs more prone to removal mediated by the recipients' reticuloendothelial system. RBCs that are nearly senescent at the time of blood collection are therefore likely to comprise the majority of cells that haemolyse over the course of the storage period, though the occurrence of haemolysis is very low, typically less than 0.8%. Biologic response modifiers (BRMs), such as cytokines, chemokines, bioactive lipids, and metabolites, accumulate during storage[52,79,80]; most of these BRMs function as pro-inflammatory agents for transfusion recipients.

Finally, processing methods and additive solutions used to prepare RCC impact the storage lesion. The buffy-coat process leads to lower haemolysis at the end of the storage period than whole blood filtration[81], and apheresis techniques generally generate more platelet-derived MPs as compared to whole blood donation[82].

Physiological consequences of transfusing RBC with storage lesions [Figure 3]

When stored RBCs are transfused to autologous healthy volunteers, a significant fraction (median 17.6%) of RBCs is cleared from circulation within 24-hr[65]. Although a small fraction of mechanically damaged cells may haemolyse intravascularly after transfusion, the majority of non-viable RBCs display eryptosis markers and are phagocytosed extravascularly by macrophages in the recipient's reticuloendothelial system[83,84]. Mechanisms of programmed cell death may activate during storage in parallel, resulting in eryptosis, which is induced by calcium influx and K^{+} efflux[23,85], cell shrinkage, exposure of PS[86] and PE[68] from inner membrane bilayer, vesiculation of MPs with loss of excess surface area[87], activation of calpains and caspases[88], and reduced deformability[89-92]. In general, the portion of removed cells increases linearly with storage duration starting from over 6% after 1 week to 11% after 6 weeks[93] in healthy volunteers, though non-linear exponential increases are observed after storage day 35, especially with respect to circulating iron metabolites, such as non-transferrin bound iron (NTBI) originating from cleared RBCs[93].

Free iron in the circulation is tightly regulated in the body not only due to its catalytic activity in ROS production, but also as the major nutrient constraining growth of siderophilic bacteria[94], a consideration relevant in patients with sepsis or bacteremia. *In vivo*, RBCs haemolysed in the circulation release iron from haeme but sequestered quickly by transferrin[95]. However, with transfusion of one unit of RBCs in healthy volunteers, up to 60 mL of damaged RBCs (25% of a unit) are removed extravascularly within 24 hours and iron is recycled. Since only 1 mL of senescent RBCs are removed hourly normally, transfusion of one unit can overwhelm both the reticuloendothelial system and the capacity of transferrin, thereby releasing NTBI into the circulation, which can result in bacterial proliferation[96]. The harmful consequences of uncontrolled NTBI in the circulation are magnified with multiple-unit transfusions and long-stored RBC units with a higher portion of non-viable cells. Additionally, for chronically transfused patients, the recipient's capacity to handle the excess iron from non-viable RBCs included in every transfused unit is overwhelmed, resulting in iron overload of tissues and subsequent organ dysfunction[97,98]. Ferryl-haemoglobin, an oxidation product of methaemoglobin by ROS, is also a proinflammatory agonist that can also cause endothelial damage[99].

NO is a signal for vasodilation that is generated by endothelial nitric oxide synthase (eNOS) near pre-capillary arterioles. Dysregulation of blood flow by disrupting NO-mediated vasoregulation is another major physiological effect of transfusing stored RBCs. Free haeme and MPs scavenge NO[100], causing INOBA and disrupting signals for increasing flow for higher oxygen delivery to hypoxic tissues. Less deformable RBCs are also implicated in causing INOBA by scanvenging NO, resulting from their tendency to flow closer to the endothelial wall, as compared to normal RBC[101]. Additionally, RBCs play a direct role in regulating their flow in capillaries via NO: RBC haemoglobin reduces nitrite in plasma to produce NO[102,103] and endothelial eNOS can be stimulated by RBC-released ATP[104-107]. Storage under conventional conditions alters the latter mechanism and the reduced glucose concentration in additive solution enables better NO production from endothelial cells stimulated by ATP release[107].

Transfused stored RBCs can provoke a pro-inflammatory response[108,109] by the cytokines,

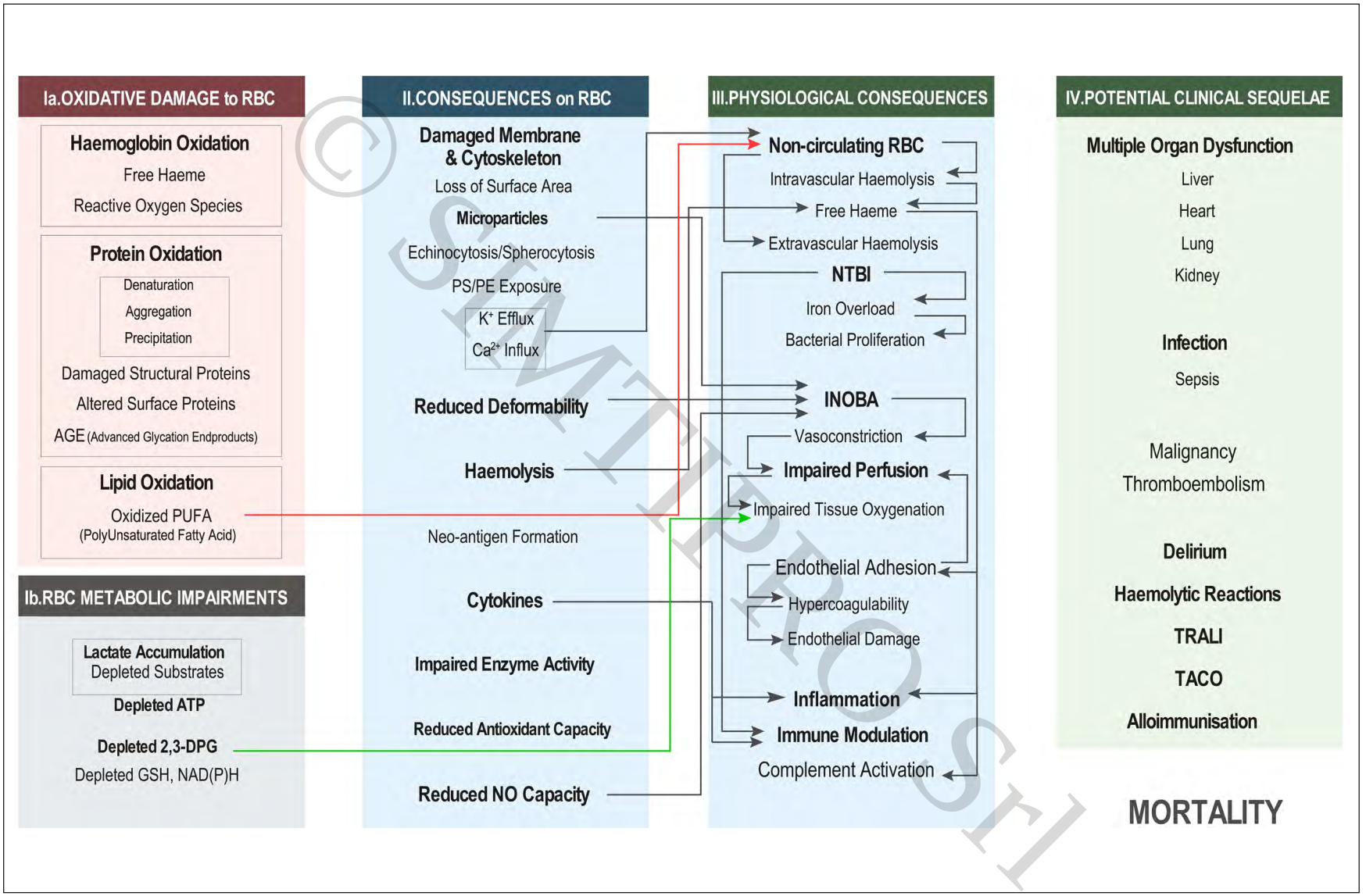

Figure 3 - Physiological consequences of specific storage lesions elucidated by *in vitro* and animal model experiments.
RBC: red blood cell; ATP: adenosine triphosphate (ATP); DPG: diphosphoglycerate; GSH: glutathione; NAD(P)H: nicotinamide adenine dinucleotide phosphate; PS: phosphatidylserine; PE: phosphatidylethanolamine; NTBI: non-transferrin bound iron; INOBA: insufficient nitric oxide bioavailability; TRALI: transfusion-related acute lung injury; TACO: transfusion-associated circulatory overload.

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eicosanoids, and free haeme within a unit, as well as the NTBI produced by extravascular haemolysis in the recipient[110]. Storage lesions also promote adhesion to endothelial cells[111,112], complement system activation[113,114], and changes in coagulability[115-117] in studies *in vitro* and in animal models. These effects also damage the endothelial lining to cause capillary leakage. In addition to the pro-inflammatory nature of stored RBCs, immune modulatory effects are also reported, attributed to NTBI[118], RBC phagocytosis, and interaction with T-cells[119].

Potential clinical sequelae and upstream linkages [Figure 4]

Panel IV of Figure 1 summarises numerous clinical outcomes suggested to be linked to transfusion of stored RBCs. Figure 4 illustrates possible linkages between elements of the three left panels to these reported clinical sequelae. It should be noted that RBC transfusion cannot be directly implicated, outside of a few exceptional cases for the entries in panel IV. Exceptions where a causal relationship can be established between a transfused RBC unit and clinical outcomes include: pathogen transmission, host *vs* graft disease, intravascular haemolytic reactions arising from mismatched RBCs, febrile responses, and some transfusion-related acute lung injury (TRALI) cases involving blood components containing incompatible plasma. For these cases, causal links to transfusion outcomes can be proven by pathogens in the donor RBCs, a high leucocyte burden, and antibodies or other BRMs in the transfused RBC unit. On the other hand, links can be only inferred between potential sequelae and transfusion of RBCs with accumulated storage lesions. The literature on this topic can be classified into four types of studies depending on how they are linked to the transfusion of stored RBCs:

A) retrospective or prospective studies examining transfusion triggers in different settings; incidences of specific morbidity are recorded as a function of a high or low transfusion trigger-where the quantity or absence of RBC transfusion is compared with specific morbidity or severity of negative outcome. A classical example is the TRICC study[120].
B) Retrospective studies in which the age of stored RBCs, as a surrogate for the extent of the storage lesion, is compared to specific morbidity or mortality outcomes. A classical example is Koch's study in cardiac patients[121] or the meta-analysis of retrospective studies conducted by a group at the NIH[122].
C) Mechanistic *in vitro* studies, where consequences of specific storage lesions are examined on suspected target cell types[107,123].
D) Animal model studies where RBCs with specific storage lesions or RBCs stored for extended time are infused into an animal prepared to simulate specific recipient conditions and overall mortality or organ-specific parameters are examined. Murine[110,119,124], canine[125], and ovine[126] models are classical examples.

Multiple organ dysfunction or individual organ failure are potential sequelae of RBC transfusion in complex surgery or in critical illness and are often used as the primary outcome measures for numerous RCTs studying the effects of transfusion trigger or storage age, a surrogate for the level of the accumulated storage lesion[14,127]. Impaired tissue oxygenation, hypercoagulability, and endothelial damage are attributed to physiological consequences of transfusing storage-damaged RBCs. Although transfusion of RBCs with storage lesions could exacerbate critical illness, it is virtually impossible to pinpoint one unit of transfused RBCs with a specific storage lesion as the major culprit. However, there is abundant literature drawing specific inferences between transfusion of stored RBCs and clinical sequelae based on clinical observations combined with results from *in vitro* experiments or animal model studies (link from Panel III to Panel IV).

- Specific organ damage, such as to the liver and heart, are attributed to iron overload in chronically transfused patients[97,128,129]. Massive intravascular haemolysis from transfusion of mismatched RBC to alloimunised patients can cause acute kidney failure[121,130-137].
- Free iron concentration in circulation is limited and tightly controlled as iron is a major limiting substrate for bacterial proliferation. Transfusing a large quantity of non-viable RBCs can easily overwhelm a recipient's ability to process excess iron, resulting in the release of NTBI into the circulation. Thus, increased infection[125,135,138-155] and sepsis[94,121,125,149,156,157] in transfused patients are attributed to bacterial proliferation arising from the availability of NTBI. Of note, extravascular haemolysis, not intravascular haemolysis, was recently associated with the transfusion of RBCs (an increased level of transferrin saturation as a hallmark) in cardiac surgery (the age of RCCs was not recorded in this observational study[158]).
- RBC transfusion reduces rate of graft rejection by recipients of organ transplants[159,160], and exhibits immunosuppressive effects, termed transfusion-telated immune modulation (TRIM)[161]. *In vitro* studies demonstrated suppression of monocyte function when incubated with stored RBCs[162,163]. Suppression of innate immunity was observed when critically ill children were transfused with RCC stored more than 21 days[164]. Those observations demonstrate immunomodulatory effects of stored

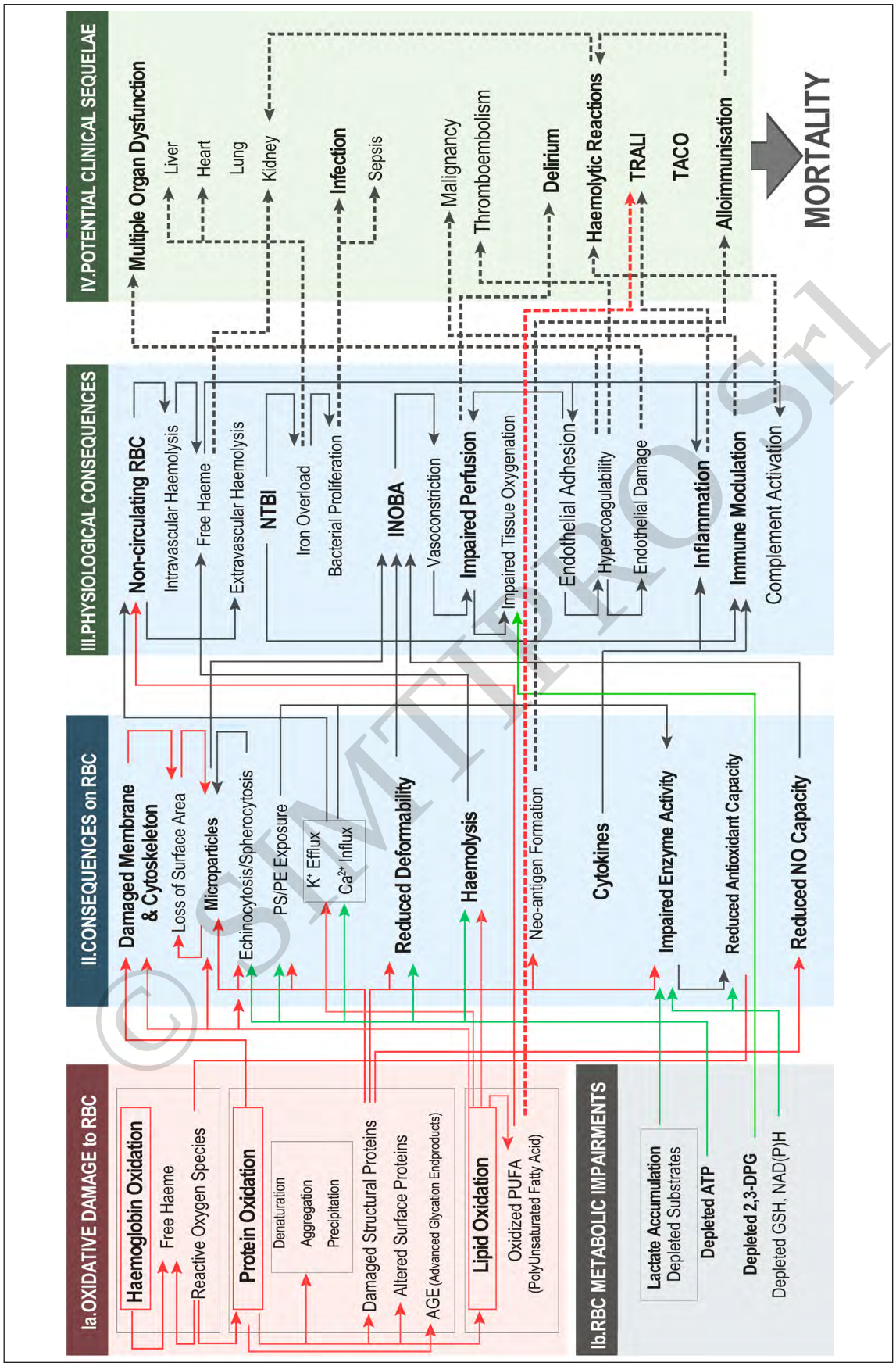

Figure 4 - Potential linkages between elements of RBC storage lesion and reported clinical sequelae of RBC transfusion.
RBC: red blood cell; ATP: adenosine triphosphate (ATP); DPG: diphosphoglycerate; GSH: glutathione; NAD(P)H: nicotinamide adenine dinucleotide phosphate; PS: phosphatidylserine; PE: phosphatidylethanolamine; NTBI: non-transferrin bound iron; INOBA: insufficient nitric oxide bioavailability; TRALI: transfusion-related acute lung injury; TACO: transfusion-associated circulatory overload.

RBCs, and link RBC transfusion to increased rate of recurring malignancy as well as infections[165].

- Transfusion of stored RBCs may exacerbate the occurrences of post-operative delirium and confusion that may be caused by impaired brain tissue perfusion and oxygen delivery[148,166-171].
- Hypercoagulability[109,115,172,173], endothelial adhesion[112,173-181], and endothelial damage attributed[182] to stored RBCs may contribute to thromboembolism in RBC transfusion recipients[142,183-185].
- TRALI involves pulmonary inflammation and can be caused by BRMs in stored RBCs, such as oxidised lipids[186-193]. The blood processing methods might have an impact since various levels of residual plasma were reported.
- Haemolytic reactions may be caused by complement activation and alloimmunisation in which neo-antigens formed in stored RBCs may contribute[194-197]; this may lead to acute kidney injury[121,130,134].
- TACO (transfusion-associated circulatory overload) is attributed to fluid overload in patients with underlying morbidity[198,199]. No links between stored RBCs quality and the incidence of TACO is apparent.
- Alloimmunisation[119,195,200-215] is often caused by transfusion of RBCs with a mismatch of minor antigens in chronically transfused patients[200,202,206-208,216], which may result in delayed haemolytic reactions[194,209]. Enhanced inflammation, stemming from transfusion of RCCs, can enhance alloimmunisation responses[203,205,217]. Additionally, modification of the RBC surface during hypothermic storage could result in erythrophagocytosis[218], which may promote alloimmunisation.

Countermeasures to reduce storage lesion

Efforts to retard haemolysis during hypothermic storage and to extend the shelf life were started at the same time as the blood banking infrastructure was established. Anticoagulant/storage solutions for whole blood were explored, culminating in approval of CPDA-1 for storage of whole blood up to 35 days. When blood component separation became the mainstay, additive solutions to replace plasma in RBC units were developed. A review by Hogman and Meryman[219] summarises efforts in the period leading up to late 1980s in which the volume, osmolality, inorganic phosphate and non-permeable ion content of additive solutions were examined to yield the additive solutions commonly used today, such as SAGM, PAGGSM, MAP, AS-1, AS-5, and AS-3. Since then, in addition to haemolysis, ATP, and 2,3-DPG levels, new parameters were increasingly measured to evaluate the quality of stored RBCs, such as microparticle release, deformability, membrane fluctuations, PS exposure, and osmotic fragility[63,220,221]. Since 2000, "omics" technologies were introduced into the field, starting with proteomics[222,223], followed by metabolomics[55,224,225], genomics[226,227] and lipidomics[28], and accompanied by system biology or *in-silico* modelling[36,228-232], greatly expanding the scope of understanding of the mechanisms underlying storage lesion progression and its genetic and epigenetic effects[228-231], as reviewed recently[55,233]. Research into better additive solutions continues to the present day in order to maintain high 2,3-DPG and ATP levels while minimising haemolysis during storage by manipulating RBC intracellular pH, either with high pH additive solution containing bicarbonate or with Donnan equilibrium employing hypotonic solution with non-permeant ions[234]. These new additive solutions remain experimental except for AS-7[235-237], which gained US FDA approval but is not available commercially. The additive solutions described above were formulated to reduce storage lesions arising from metabolic impairments; the subsequent consequences that are suggested in Figure 4. In parallel to developing RBC additive solutions, rejuvenation solutions based on pyruvate, inosine, phosphate and adenine, yielding levels of 2,3-DPG and ATP comparable to freshly collected RBCs when processed at the end of storage[238,239], are available. More recently, adding such solutions was shown to be effective during hypothermic storage[240,241] by reactivating critical metabolic pathways of stored RBCs[241].

Addressing metabolic impairments by adjusting additive composition and pH positively affected maintenance of RBC energetics as well as levels of some antioxidant metabolites. However, since biochemical reactions are limited during hypothermic storage, reducing metabolic impairments alone may not fully reduce storage lesions caused by oxidative stress. Diffusion of oxygen from ambient air through the storage bag coupled with its higher solubility at storage temperature leads to an increase in oxygen concentration, the main reactant in oxidative reactions. Thus, in addition to addressing metabolic impairments, provisions to reduce the direct sources of oxidative damage should be included in comprehensive solutions to reduce the development of the RBC storage as illustrated in Figure 4, where oxidative damage due to O_2 (Panel Ia) affects every item downstream (Panel II-IV).

Two general approaches were proposed to reduce oxidative damage during hypothermic storage: i) inclusion of anti-oxidants in the additive solution; and ii) reduction of pro-oxidants in stored RBCs by hypoxic storage. Chemicals such as nicotinic acid[242], melatonin[243], L-carnosine[244], ascorbic acid[245,246], quercetin[247], iron chelators[248], and N-acetylcysteine[249,250] have been suggested as antioxidants to be included in additive solutions, but the improvements appeared insufficient and none has been tested extensively for commercial production. Supplementation with

antioxidants, such as ascorbic acid (which can only be up taken by RBCs in its oxidised form, dehydroascorbate) and/or N-acetylcysteine, while boosting RBC antioxidant capacity[246,250,251], ended up depleting glutathione pools by favoring reduced glutathione conversion to disulfide during storage[250] and limiting energy metabolism. The latter is attributed to the competitive uptake of dehydroascorbate by RBCs via the transporter GLUT1 at the expense of glucose[51]. Alternative additives, including non-glucose sugars such as fructose and mannose[225], in the formulation may obviate this problem. Lipophilic antioxidants, such as vitamin E, may be valuable alternatives to specifically mitigate lipid peroxidation[252,253], but they also induce morphological changes.

Hypoxic storage, where the oxygen content of RBC units is reduced to low levels (e.g., less than 4% oxy-haemoglobin [$\%SO_2$][254]) prior to refrigeration and maintained throughout storage, was proposed as an alternative to antioxidant-based additive solutions to reduce oxidative stress during hypothermic RBC storage[254-259]. The rationale for implementing hypoxic storage is to reduce oxygen, the essential substrate for haemoglobin oxidation that generates a multitude of ROS as byproducts. Additionally, oxygen is required for sustaining the lipid peroxidation cycle catalysed by ROS.

Hypoxic storage was shown to counteract some metabolic impairments without requiring novel additive ingredients[256] independent of the reduction in oxidative stress. During pre-storage processing to reduce oxygen content in the RCC, carbon dioxide is also reduced. Carbon dioxide depletion increases cytosolic pH that was lowered from its physiological level by exposure to acidic anticoagulant and additive solutions. The resulting more neutral pH in the early phase of storage results in sustained flux through the glycolytic pathway and elevated 2,3-DPG levels, which are highly sensitive to pH and normally depleted early during hypothermic storage[256,260]. Additionally, deoxyhaemoglobin causes metabolic modulation to release glycolytic enzymes, such as phosphofluctokinase and glyceraldehyde dehydrogenase, sequestered at the band 3 binding domain, thereby enhancing overall glycolytic flux during hypoxic storage[44,261,262]. Under reduced oxygen concentration, hexokinase output to the PPP is partially blocked due to metabolic modulation as well as limiting the concentration of $NADP^+$ (limiting substrate for PPP), which may have resulted in curtailed glutathione levels[260].

Factors affecting storage lesion development: donor characteristics and RCC preparation methods

RBC responses to hypothermic storage, such as maintenance of glutathione levels and the extent of haemolysis, are heritable traits and depend on genetic makeup of the donors[227,263-265]. Evolutionary selection pressure in different geographical environments resulted in multiple strategies to optimise human survival, which included subtle differences in RBC physiology, resulting in a wide variation of RBCs in the current-day blood donors. For example, selection against malaria resulted in various mutations providing a survival advantage in heterozygous carriers, but their RBCs may be less suitable for hypothermic storage[266-268] or less efficacious for transfusion in specific patient categories[269]. An additional large variability is the oxygen saturation of the collected whole blood, resulting in a wide distribution of oxygen content in the prepared RCCs prior to hypothermic storage. Although RCC preparation procedures, donor gender, and the elevation of blood donation sites affect median percent haemoglobin oxygen saturation ($\%SO_2$), a very wide $\%SO_2$ distribution ranging from below 20% to above 80% was observed[270,271]. This variation in the initial oxygen content of RCCs could contribute to overall variability of stored RCC quality, since oxygen is the major substrate for oxidative reactions resulting in oxidative storage lesions, and oxygen concentration profoundly affects RBC metabolism during hypothermic storage[44,51,261].

The specific method used to prepare RCCs from donated whole blood for hypothermic storage, independent of donor-dependent factors (gender, donor age, iron status etc.), affects the characteristics of stored RBCs[81,272,273,500] and may contribute to non-uniform clinical outcomes[274]. There are various procedures in blood donation (whole blood or apheresis), leucocyte filtration (whole blood, RBC, no filtration), component preparation (buffy coat, hard or soft spin), additive solutions (AS-1, AS-3, AS-5, SAGM, MAP, PAGGSM), and process time before refrigeration (8 hours or 24 hours after overnight room temperature hold). Leucocyte filtration could positively affect the quality of stored RBC by reducing ROS produced in leucocytes[275]. Gamma irradiation[276-281] and pathogen inactivation processes[282-286] generate ROS, causing oxidative damage and exacerbating the rate of storage lesion development during subsequent hypothermic storage. Washing RCCs removes damaged RBCs and other potentially harmful byproducts, such as potassium, MPs, and cytokines[d,178,287-289]. Aliquoting a single RCC unit throughout its shelf life for repeated transfusion in neonates is acceptable in order to limit multiple donor exposure[290]. Cryopreservation overcomes the 6-7 week storage limit of refrigerated RCCs by deep-freezing RBC in cryoprotectant solutions containing glycerol[291].

[d] Negative outcome was reported with washing older RCCs, presumably from inflicting mechanical damage on older fragile RBCs during the process[155].

This technology was developed in 1950s-1980s, as summarised in early[292] and more recent[293] reviews. Subsequent development of a closed deglycerolisation system allows for extended storage duration of post-thaw RCC from 24 hours to 14 days[294]. Numerous publications provide a detailed characterisation of thawed RBCs including rheologic properties[295], microvesiculation[296], and their potential superiority over conventionally stored RBCs in trauma settings[297]. Cryopreserved RCC is routinely used for rare blood types and in military settings in the United States of America and Europe.

Quality of stored RBCs: results from randomised controlled trials on age of blood and their implications

In nearly all cases, the damage RBCs sustain while stored hypothermically *ex vivo* accumulates throughout the storage period, albeit at different rates depending on the genetic makeup, and possibly, the dietary and environmental exposure of each donor. Therefore, to evaluate the clinical consequences of transfusing RBCs with storage lesions, numerous RCTs have taken place, including five large scale ones in the past five years[10-14] and two smaller trials earlier[15,298], all using the age of stored RBCs as a surrogate for the extent of the accumulated storage lesion. These large studies compared fresh (6-12 days on average) to standard-issue or moderately aged RBC units (~3 weeks; except for one study at 5 weeks[12]) under various patient conditions and found no differences in mortality or the development of selected morbidities, indicating that there is no inferiority in transfusing RBCs using standard practice (oldest units available) when compared to transfusing fresher RBCs (freshest available). For details, readers are referred to the original reports, several meta-analyses[8,9,299-301], and recent commentaries[7,302]. These results dispelled blood establishments' potentially critical safety concerns for recipients of their products. However, the debate is still open on transfusion of end-of-storage RCCs (older than 28 or 35 days)[16,303-305], since the outcomes of patients receiving a large quantity of old RCCs on one occasion or those receiving transfusions chronically over an extended period of time have not been examined by RCTs[e]. Additionally, different RBC manufacturing methods or donor factors could affect the quality of stored RBCs[81,274,285,306,307], potentially confounding the results of multicentered RCTs. Therefore, questions of safety remain for vulnerable patient populations receiving transfusions of RCCs with a high storage lesion burden.

[e]A secondary analysis of data from an RCT (PROPPR trial on trauma) suggested an association between transfusion of 10 or more units of RCCs older than 22 days, and an increased liklihood of mortality within 24 hours[501].

Conclusions and future directions

Placed outside of the donor's circulation and stored in a blood bank refrigerator, RBCs incur storage lesions. The recent application of "omics" technologies has made available vast quantities of new information, providing new paths to formulate, reduce, and test hypotheses on the mechanisms of storage lesion development. However, even with the current relative abundance of data, such an undertaking would not be easy as there is wide genetic variability of performance in donors' RBC under hypothermic storage conditions[29,81,306,308,309]. "Omics" studies scopes need to expand from a small number of subjects currently examined in detail to a much larger and diverse population. Different blood processing strategies need to be considered to optimise the function of donors' characteristics and patients' needs, keeping in mind the economic, technical, and logistical issues, and that our donors are of primary importance to sustain the transfusion chain[310,311]. As reviewed in previous sections, a body of evidence exists in animal models and humans suggesting that physiological responses to transfusion of damaged RBCs are adversely affecting the recipients. However, a direct link between transfusion of RBCs with specific storage lesions and observed negative outcomes in diverse recipients with different reasons for requiring transfusion therapy is difficult to establish unequivocally. This challenge is further complicated by pre-existing comorbidity as well as genetic variability in recipient responses to transfusion.

A large number of individuals will still receive stored RBCs (e.g. 4-5 million patients annually in the USA alone), even though RCC consumption is steadily declining[f,312], and the potential harmful effects of transfusions with high storage lesion burden remains a concern. Additionally, selected categories of patients are potentially more vulnerable: patients with less common blood groups (e.g. AB) often receive older RCCs as compared to other groups, and patients who are massively or chronically transfused receive a disproportionately large fraction of RBC units that may include older RCC. Especially for the latter patients, the potential sequelae of transfusion could be amplified because of high levels of exposure to RBCs stored over an extended period. The published large-scale RCTs on "age of RBC" do not provide objective evidence to assure safety of exposure to RBCs with high storage lesion burden in massive or chronic transfusion recipients. Because of these considerations, continued efforts to improve RBC processing/storage methods in order to reduce the storage lesion should benefit recipients and improve the overall cost-effectiveness of the patient-care system.

[f]Shifing demographics might change this trend in a decade[313].

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Diclosure of conflicts of interest

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