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Screening of the Effects of Additive Solutions on the Storability of Packed Cord Blood Erythrocytes

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Abstract

Preterm infants frequently develop anaemia and require repeated red blood cell (RBC) transfusions. Currently, adult donor erythrocytes are used for this purpose. However, neonatal erythrocytes may represent a more physiologically appropriate alternative.

In this study, isolated adult and umbilical cord blood (CB) erythrocytes were stored for 28 days in seven different additive solutions (SAGM, AS1, AS3, AS5, AS7, PAGSM, and PAG3M) using a small-scale ex vivo storage model. Haemolysis, glucose consumption, lactate production, and morphological changes were assessed over time.

Storage solution had a strong impact on haemolysis and metabolic parameters, whereas the effect of cell origin was generally minor. SAGM consistently induced the highest haemolysis in both groups, while alkaline and phosphate-containing solutions (AS7, PAGSM, PAG3M) showed improved storage performance. Biochemical parameters followed patterns similar to haemolysis, supporting the dominant role of additive solution composition. In contrast, morphological analysis revealed more pronounced differences between adult and CB erythrocytes. While haemolysis patterns were largely comparable, CB erythrocytes showed distinct morphological alterations. Notably, haemolysis did not consistently correlate with sphero-echinocyte formation in CB samples, indicating partially uncoupled biochemical and morphological additive lesions.

These findings suggest that additive solution composition is the primary determinant of RBC storage quality, while CB erythrocytes display distinct morphological behaviour likely reflecting intrinsic biological differences. Importantly, conditions that performed well in adult erythrocytes also performed well in CB cells, supporting their translational applicability.

Zusammenfassung

Frühgeborene entwickeln häufig eine Anämie und benötigen wiederholt Erythrozytenkonzentrate. Derzeit werden adulte Spenderzellen verwendet, obwohl neonatale Erythrozyten eine physiologisch geeignetere Alternative sein könnten.

In dieser Studie wurden adulte und Nabelschnur-Erythrozyten über 28 Tage in sieben Additivlösungen (SAGM, AS1, AS3, AS5, AS7, PAGSM, PAG3M) im Small-Scale-Modell gelagert. Hämolyse, Glukoseverbrauch, Laktatbildung und Morphologie wurden im Verlauf analysiert.

Die Additivlösung determinierte Hämolyse und Metabolismus maßgeblich, während die Zellherkunft kaum eine Rolle spielte. SAGM induzierte in beiden Gruppen die höchste Hämolyse, wohingegen alkalische und phosphathaltige Medien (AS7, PAGSM, PAG3M) die Lagerungsqualität verbesserten. Die metabolischen Parameter spiegelten diese Hämolysemuster wider.

Im Gegensatz dazu zeigten sich morphologisch deutliche Unterschiede: CB-Erythrozyten wiesen spezifische strukturelle Veränderungen auf. Da die Hämolyse in CB-Proben nicht mit der Formation von Sphäro-Echinozyten korrelierte, weisen biochemische und morphologische Lagerungsschäden hier eine teilweise Entkopplung auf.

Zusammenfassend ist die Additivlösung der Hauptfaktor für die metabolische Stabilität, während CB-Erythrozyten ein eigenständiges morphologisches Verhalten zeigen. Da für adulte Zellen optimierte Bedingungen auch bei CB-Zellen überzeugten, unterstützt dies deren translationale Anwendbarkeit.

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Abbreviation

2,3 DPG	2,3 Diphosphoglycerate
AGM	Aorta–gonad–mesonephros
AI	Artificial Intelligence
AMG	Arzneimittelgesetz
AOP	Anaemia of prematurity
AS1,3,5,7	Additive solution 1,3,5,7
ATP	Adenosine triphosphate
AUC	Area under the curve
BASG	Bundesamt für Sicherheit im Gesundheitswesen
BMI	Body Mass Index
BPD	Bronchpulmonary dysplasia
BSG	Blutsicherheitesgesetz
CPD-A	Citarte phosphat dextrose adenine
ELGAN	Extremely low gestational age neonate
EMA	European Medicine Agency
IVH	intraventricular haemorrhage
FDA	U.S Food and Drug Administration
G6PD	Glucose-6-phosphate dehydrogenase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSSG	Glutathione disulfide
HbA	Adult Haemoglobin
HbF	Foetal haemoglobin
LN_2	Liquid nitrogen
MAP	Mannitol adenine phosphate
MCV	Mean corpuscular volume
MCHC	Mean corpuscular haemoglobin concentration
NADPH	Nicotinamide adenine dinucleotide phosphate
NEC	Necrotising enterocolitis

NICU	Neonatal intensive care unit
NO	Nitric oxide
PAG3M	Phosphate-Adenine-Glucose-Guanosine-Gluconate Mannitol
PAGSM	Phosphate-Adenine-Glucose-Guanosine-Saline Mannitol
PFK	Phosphofructokinase
PMA	Postmenstrual age
PPP	Pentose phosphate pathway
pRBC	Packed red blood cells
RBC	Red blood cells
RDW	Red cell distribution width
ROP	Retinopathy of prematurity
ROS	Reactive oxygen species
SAGM	Saline adenine glucose mannitol
SN	Supernatant
SNO-HbF	S-nitroso foetal haemoglobin
TCA	Trichloroacetic acid
WBC	White blood cells
WHO	World Health Organisation

Content

1 Introduction

1.1 Pharmaceutical and regulatory aspects of red blood cell products

In the EU, human blood and blood components are regulated by specific directives rather than by general pharmaceutical legislation. Of particular importance is Directive 2002/98/EC (the EU Blood Directive), which establishes minimum standards for the collection, testing, processing, storage and distribution of whole blood and blood components (1).

In Austria, blood and blood products are classified as medicinal products under the Medicines Act (AMG). In addition, the Blood Safety Act 1999 (BSG 1999) regulates the collection and testing of human blood and blood components. According to Section 1 of the BSG, blood and blood components must be collected and tested in a manner that ensures the best possible protection for donors and patients (2). In addition, there are, among others, the hemovigilance regulation, which makes the reporting of adverse reactions mandatory, the Blood Donor Regulation for donor criteria, and a Quality Assurance Regulation, which outlines the quality management system for blood donation establishments. Responsibility for implementation and supervision lies with the Federal Office for Safety in Healthcare (BASG), which is responsible, within the framework of the AMG/BSG, for authorising and inspecting blood donation facilities and plasma centres, as well as coordinating hemovigilance (3).

In Austria, approximately 340.000–365.000 units of blood are transfused annually, with approximately three-quarters constituting packed red blood cells (pRBCs). This corresponds to roughly 250.000–300.000 pRBC transfusion per year in Austria (4). In the EU, around 22 million units of blood are collected annually from 15 million donors (5). Each of these whole blood donation is then separated into units of packed pRBC, platelets and plasma, further highlighting the sheer volume of blood processing and blood allocation (5).

Within Europe, haematological and oncological patients are consistently the most heavily transfused populations and account for approximately 30% of all transfused units (6). They are closely followed by surgical patients, particularly those undergoing

cardiac and gastrointestinal surgery as well as patients undergoing critical care treatments (6). Across all indications, elderly patients represent the largest recipient group. Approximately 66% of all transfusion recipients are over 65 years of age, and nearly 50% of all RBC units are transfused to patients over 70 (7). This likely reflects the higher prevalence of anaemia-associated conditions and the increased surgical complexity in this age group (7). Sex-related differences in transfusion propensity are observed, with women predominating among recipients aged 30–54 years due to obstetric and gynaecological indications, and among those older than 90 years because of their greater life expectancy. (7). Despite premature babies account for only a relatively small proportion of the total number of red blood cell transfusions administered, they represent a disproportionately transfusion-dependent population group. In a large prospective European multicentre study across 64 neonatal intensive care units (NICUs) in 22 countries, 36.5% of preterm infants born before 32 weeks of gestational age received at least one RBC transfusion within the first 28 days of life. Moreover, transfusion rates exceeded 90% among extremely low gestational age neonates (8).

Given the disproportionately high transfusion burden in preterm infants and the physiological distinctiveness of neonatal erythrocytes as well as the vulnerability of immature organ systems to transfusion-associated complications, understanding transfusion requirements and risks in preterm infants is of utmost clinical and pharmaceutical relevance.

1.2 RBC transfusion in preterm infants

According to the World Health Organisation (WHO), preterm birth is defined as the delivery of a live-born infant before 37 completed weeks of gestation (9) and accounts for approximately 10% of all births worldwide (10). It continues to be one of the most common causes of death in children under the age of five (11).

Preterm birth is associated with a range of short- and long-term health complications. Immediate complications include hypoxaemia (12) and anaemia (13), followed by bronchopulmonary dysplasia (BPD) (14), retinopathy of prematurity (ROP) (15), intraventricular haemorrhage (IVH) (16), necrotising enterocolitis (NEC) (17),

periventricular leukomalacia (PVL) (18), and post-haemorrhagic hydrocephalus (PHVD) (19). Long-term consequences may include impairments in cognition, behaviour, motor function, vision, and hearing (20), particularly in infants with very low (<1500 g) or extremely low (<1000 g) birth weight (11).

All newborns experience a physiological decline in haemoglobin (Hb) during the first months of life. This process is facilitated by a less effective endogenous erythropoietin production, a shorter life span of erythrocytes and rapid postnatal growth, which leads to a dilutional effect due to increased circulating blood volume (20). Early umbilical cord clamping may increase the risk of iron deficiency anaemia in both term and preterm infants. Moreover, phlebotomy can contribute to the development of anaemia (21). While physiological anaemia is generally well tolerated in term infants, it is more pronounced and occurs earlier in preterm infants. In term infants, haemoglobin reaches a minimum value of 10.3 g/dL, whereas in preterm infants, levels may decrease to as low as 7.1 g/dL within four to eight weeks after birth (20). This exaggerated decline in haemoglobin levels in preterm infants is commonly referred to as anaemia of prematurity (AOP; ICD-10 code P61.2) (13,22). It represents one of the most common haematological complications in preterm infants, particularly in very low birth weight and extremely low birth weight neonates, where the incidence of neonatal anaemia can reach up to 41% and 60%, respectively (13).

Management and prevention of AOP include both clinical strategies and interventional therapies. Early umbilical cord clamping may increase the risk of iron deficiency anaemia in both term and preterm infants. Güner and Saydam demonstrated improved haematological parameters after delayed cord clamping in term infants (23). Similar benefits were also reported for preterm infants, including reduced hospital mortality, higher haematocrit levels, and a lower need for blood transfusions following delayed cord clamping (24). The standard treatment for AOP in preterm infants is red blood cells transfusion (21). RBC transfusions are life-saving interventions that improve oxygen delivery. They increase haemoglobin and haematocrit levels, thereby enhancing oxygen transport capacity and reducing episodes of apnoea and hypoxaemia, while mitigating the adverse effects of anaemia on cerebral oxygenation (11). Consistent with this, transfusion with packed red blood cells has been shown to significantly reduce periodic breathing, apnoea episodes, apnoea density, and heart

rate in anaemic preterm infants (25). However, RBC transfusions have also been associated with adverse outcomes, including NEC (26), ROP (26), BPB dysplasia (26), IVH (26), and long-term neurodevelopmental impairment. (20). This occurs due to differences between adult and neonatal red blood cells.

1.3 Adult vs neonatal erythrocytes

Neonatal erythrocytes differ from adult erythrocytes in size, shape, membrane properties, cellular metabolism, haemoglobin composition, oxygen transport and lifespan (27).

Neonatal erythrocytes are significantly larger, with size and volume decreasing throughout gestation (28) and after birth until adult values are reached at around one year (29). Adult erythrocytes are biconcave discs with a diameter of 7.5–8.7 μm (30), whereas neonatal erythrocytes show greater heterogeneity in shape, including dysmorphic forms such as echinocytes, acanthocytes and dacryocytes, as well as a higher proportion of reticulocytes (16). Moreover, small numbers of nucleated red blood cells can be present in neonatal peripheral blood during the first hours after birth (32).

Neonatal erythrocyte membranes differ from their adult counterpart in lipid composition and exhibit alterations in membrane fluidity and transport activity (27,29). Studies have suggested that neonatal erythrocyte membranes harbour comparatively high amounts of cholesterol and phospholipids, as well as palmitic and arachidonic acid and markedly lower levels of linoleic acid, when compared to adult cells (33). As a result, they have a larger surface area to volume ratio, which has been suggested to underlie their suggested increased resistance to osmotic stress (29). Moreover, they show a higher level of polyunsaturated fatty acids (34). However, the lipid profile of neonatal erythrocytes seems highly dependent on the mother's diet, genetic factors and placental transfer (27). Regarding membrane protein composition, no fundamental qualitative differences in the membrane proteome have been observed. Nevertheless, quantitative differences in the expression of individual proteins are evident between neonatal and adult cells. In adult erythrocytes, carbonic anhydrase 1 and 2 and aquaporin 1 are expressed more strongly, whereas neonatal erythrocytes show higher

expression of certain myosin subunits (35). Furthermore, neonatal erythrocytes have a more strongly negatively charged cell surface, as they contain more sialic acid (27).

In addition to structural differences, neonatal and adult erythrocytes also exhibit functional differences. Neonatal cells have lower channel-mediated water permeability, but show increased activity of the K+/Cl- cotransporter (36). The data on osmotic fragility remain inconclusive. Under moderate hypotonic stress, neonatal erythrocytes, especially those from premature infants, appear to be more resistant to haemolysis than adult cells. However, umbilical cord erythrocytes show greater heterogeneity and seem to be more sensitive to volume changes under extreme osmotic conditions (27).

Moreover, there are differences in the deformation and flow characteristics of erythrocytes (37). Neonatal red blood cells show a lower resistance to stretching and folding, a slower return to their original shape and reduced filterability through narrow pores (37,38). These differences are primarily related to the increased size of neonatal red blood cells and are not attributable to alterations in membrane or haemoglobin viscosity, as both are generally comparable to those of adult cells (27). Under continuous mechanical stress, erythrocytes eventually reach a point of irreversible membrane damage (39). Although the critical shear force leading to membrane failure is similar in neonatal and adult RBCs, membrane rupture occurs more quickly in neonatal cells once this threshold is exceeded (39). In addition, a subpopulation of particularly rigid and dense cells exists, presumably due to membrane loss and increased intracellular viscosity (40). These factors may contribute to the increased mechanical fragility and shorter lifespan of neonatal erythrocytes (27).

Differences are also observed in aggregation behaviour (27). Rouleaux formation is significantly reduced, especially in premature infants. This is partly due to lower plasma viscosity and the composition of neonatal plasma, but also influenced by cell-intrinsic properties (41). These rheological characteristics are clinically relevant, as erythrocyte deformability affects survival time in the circulation, tissue oxygenation and transfusion outcome (27). The transfusion of adult donor erythrocytes, which are less deformable than neonatal or umbilical cord blood RBCs, may therefore alter the rheological balance in the neonatal circulation (27).

The most conspicuous difference between neonatal and adult erythrocytes is the composition of haemoglobin. While HbF is made up of two alpha and two gamma chains, HbA is composed of two alpha and two beta chains (42). During pregnancy, HbF is dominant. Around the time of birth (40 weeks of gestation), the physiological transition to HbA begins, during which γ-chain production decreases and β-chain production increases (43). Even though the HbF production after birth decreases, there is still a small part that produces HbF during adulthood. While coinciding with it, the switch from HbF to HbA is not stimulated by birth itself. In the first weeks of life, premature babies have HbF levels that correspond to those of foetuses at the same stage of development. Only when they reach a post-conceptual age of around 38 weeks do their HbA levels become similar to those of full-term babies (27). Thus, the switch from HbF to HbA seems to be a developmental phenomenon rather than a stimulus dependent. Functionally, HbF differs significantly from HbA. HbF binds oxygen more strongly, likely because its γ chains interact less effectively with 2,3-DPG. This allows the foetus to take up oxygen from the mother's blood (42). In addition, haemoglobin reacts quickly with nitric oxide (NO). RBCs can produce and release NO themselves, which contributes to the regulation of microcirculation (27). There is evidence that HbF enables higher NO availability, for example, through increased nitrite reductase activity or faster oxidative denitrosylation (44). Premature infants with high HbF levels show increased S-nitrosohemoglobin (SNO-HbF) levels, which may contribute to vascular regulation. HbF demonstrates greater structural stability and tetrameric integrity, resulting in reduced haem degradation and lower free iron release. Additionally, it exhibits lower cytotoxicity toward endothelial cells and reduced reactive oxygen species formation, attributed to its higher pseudoperoxidase activity. Its stronger pseudoperoxidase activity protects neonatal RBCs from oxidative stress. In addition, HbF influences the energy metabolism of the cell through its binding properties (27).

Neonatal RBCs differ from adult RBCs in their metabolic function (27). Mature erythrocytes rely exclusively on glycolysis for ATP (Adenosine triposphate) production due to the absence of mitochondria (45). Thus it is interesting to note that cord blood RBCs exhibit distinct alterations in glycolytic and related antioxidant pathways (46). Compared to adult RBCs, neonatal cells show increased activities of enolase and phosphoglycerate kinase, but reduced phosphofructokinase activity, resulting in

altered levels of glycolytic intermediates and differences in 2,3-diphosphoglycerate (2,3-DPG) metabolism (27). In addition, glucose-6-phosphate dehydrogenase (G6PD) activity is higher in neonatal RBCs (47), indicating enhanced PPP (pentose phosphate pathway) activity and increased NADPH (nicotinamide adenine dinucleotide phosphate) generation for protection against oxidative stress (27). Furthermore, HbF interacts more weakly with band 3 than adult haemoglobin HbA (48), which may alter the balance between glycolysis and the pentose phosphate pathway during hypoxia and refrigerated storage (27). Although cord blood contains higher reticulocyte counts than adult blood (49), studies comparing neonatal RBCs with reticulocyte-rich adult RBCs demonstrated that the observed metabolic differences cannot be explained by reticulocytosis alone (27).

Adult red blood cells originate in the bone marrow, where erythropoietin stimulates erythroid progenitor cells to differentiate into erythrocytes. Mature erythrocytes then circulate for a maximum time of 120 days before being removed by macrophages, mainly in the spleen (50). During aging, erythrocytes gradually lose membrane elasticity and deformability and intracellular enzyme systems involved in protection against oxidative stress become progressively depleted, leading to the accumulation of cellular damage (50). In contrast, neonatal erythropoiesis occurs in several developmental waves and shifts between different anatomical sites during gestation, including the yolk sac, the aorta–gonad–mesonephros (AGM) region, the neonatal liver, spleen, and finally the bone marrow (51). Especially in extremely preterm infants, erythropoiesis may still occur in the neonatal liver, resulting in the presence of more immature erythroid cell populations and increased reticulocyte counts compared to adults (27). In addition, preterm infants often have limited iron stores due to the interruption of placental iron transfer during late gestation (52). Corresponding to these developmental differences, neonatal erythrocytes also exhibit a reduced lifespan of approximately 60–90 days in term neonates, compared to adult erythrocytes. This shortened lifespan is attributed to intrinsic properties of neonatal erythrocytes, including accelerated membrane loss, increased susceptibility to oxidative stress, and altered metabolic and enzymatic stability compared to adult erythrocytes (27).

Given the considerable biological and functional differences between foetal and adult erythrocytes, these distinctions may have important implications in the context of red

blood cell transfusion. Foetal erythrocytes differ in several key aspects, including haemoglobin composition, metabolic activity, and membrane properties, all of which may influence their behaviour during storage and after transfusion. These differences raise the question of whether the currently used adult donor blood is the most appropriate transfusion product for preterm infants. Therefore, the potential advantages of using cord blood–derived erythrocytes as an alternative transfusion source have gained increasing attention and warrant further investigation.

Category	Neonatal Erythrocytes	Adult Erythrocytes
Size & morphology	Larger cell volume, increased RDW, dysmorphic shapes, higher reticulocyte count	Stable cell volume, predominantly biconcave discocytes
Membrane composition	Higher cholesterol/phospholipid content; altered fatty acid profile; higher membrane charge due to more sialic acid.	Relatively higher linoleic acid content; lower surface charge.
Mechanical properties	Reduced deformability, increased mechanical fragility	Higher deformability and mechanical stability
Haemoglobin	Predominantly HbF ($\alpha_2\gamma_2$); higher oxygen affinity and oxidative stability	Predominantly HbA ($\alpha_2\beta_2$)
Metabolism	Altered glycolysis/PPP balance, altered redox regulation	Stable glycolytic metabolism and redox homeostasis
Oxidative stress	Higher G6PD activity; enhanced PPP/NADPH generation; HbF releases less haem and free iron; fewer free radicals; stronger pseudoperoxidase activity	Oxidative damage accumulates with cell ageing, progressive depletion of antioxidant systems, increased membrane damage and reduced deformability over time
Lifespan	~60–90 days (term)	~120 days

Table 1: *Overview of the major structural, functional and metabolic differences between neonatal and adult erythrocytes. Adapted from Pellegrino et al., 2024 and Orkin et al., 2015 (27,29)*

1.4 Cord blood transfusion for preterm infants

Despite its widespread use in neonatal intensive care, transfusion of adult erythrocytes has been associated with several complications, including BDP (26) and ROP (26). These associations are thought to be partly related to differences in haemoglobin composition between adult and neonatal erythrocytes. Transfusion of adult erythrocytes alters the proportion of neonatal haemoglobin (HbF) in the circulation of preterm infants. Because HbF has a higher affinity for oxygen than HbA, the oxygen supply to the tissue is different (53). This increased peripheral availability of oxygen may cause oxidative damage in premature tissue, especially because the antioxidant system is not yet fully developed (54). Moreover, repeated red blood cell transfusion may lead to increased free iron levels, which favour the production of free oxygen radicals and thus the development of ROP and BPD (55).

The best way to mitigate risks associated with red blood cells transfusion is prevention. There are several ways to reduce the risk of anaemia in premature babies and to lower the likelihood for receiving a blood transfusion. One approach is delayed umbilical cord clamping. Güner and Saydam demonstrated that delayed cord clamping for more than one minute after birth allows continued placental blood transfer, resulting in significantly increased haematocrit and haemoglobin levels, both at 48 hours of life and at a four-month anaemia screening (23). In addition, minimising the amount of blood drawn for laboratory tests also helps to reduce iatrogenic blood loss. Counsilman et al. found that extremely premature infants lost a median of 24.2 mL/kg, equivalent to 28.5% of their total blood volume, through phlebotomy for laboratory testing alone during the first four weeks of life, with losses being significantly higher in the most immature infants (56). Thus, reduction of blood test frequency as well as the usage of modern, low blood volume requiring point of care devices could significantly mitigate this burden. Furthermore, the body's own erythropoiesis can be supported by adequate iron supplementation, which is routinely administered in nearly all European NICUs from two weeks of age (57). Although erythropoietin supplementation can reduce transfusion requirements, it has not been shown to improve long-term neurodevelopmental outcomes in large randomised trials (58,59), and is therefore only used routinely in approximately 22% of European NICUs (57).

More recently proposed alternatives to the transfusion of adult RBCs are autologous and allogeneic CB-RBC transfusion. In allogenic transfusion, umbilical cord blood, obtained immediately after birth, is processed and autologously transfused into the infant. Although data on the use of autologous CB in preterm infants remain limited, existing studies suggest that this approach is safe. However, the volume of blood is only sufficient for approximately one to two transfusions (60). Alternatively, current studies are investigating the safety and efficacy of transfusion of allogenic umbilical cord blood, derived from term-born infants. The most prominent study on this topic is the BORN study (61), a multicentre, double-blinded, randomised controlled trial recruiting extremely low gestational age neonates (ELGANs) born between 24+0 and 27+6 weeks of gestation across 10 Italian NICUs, with a planned total sample size of 146 patients (73 per arm). In this trial, patients are randomised 1:1 to receive either conventional adult donor RBCs or allogeneic cord blood-derived RBCs from birth until 31+6 weeks postmenstrual age (PMA). The primary outcome is the incidence of severe ROP at 40 weeks PMA or discharge. Secondary outcomes include ROP requiring treatment, BPD, and a composite outcome of death, severe ROP, BPD, and NEC. In a prespecified interim analysis of the first 58 patients, cord blood RBC transfusions were confirmed to be safe, with similar overall incidence and severity of adverse events across both arms. However, patients receiving exclusively adult RBCs or a combination of both blood types showed more severe forms of bradycardia, pulmonary hypertension, and haemodynamically significant patent ductus arteriosus in the per-protocol analysis. Each adult RBC transfusion carried a relative risk for severe ROP of 1.66 % compared to CB-RBCs. Furthermore, analysis of the HbF area under the curve suggested that HbF decline before 30 weeks PMA is particularly critical for the development of severe ROP, and that subsequent CB-RBC transfusions administered after this window may not sufficiently mitigate this risk, underscoring the importance of early adoption of a CB-RBC transfusion strategy. It should be noted that the interim analysis was limited by a high rate of protocol deviations, with 104 A-RBC units transfused compared to only 49 CB-RBC units, partly reflecting challenges in CB unit availability. The full trial results are pending (61,62).

While these findings highlight the potential of CB RBC transfusions as a promising alternative to adult donor blood, an equally important yet underexplored consideration is the conditions under which red blood cells are processed and stored prior to

transfusion. In the BORN trial, CB RBCs were stored in saline-adenine-glucose-mannitol (SAGM) (62), which is the standard additive solution used across European blood banks (63). Despite the profound biological differences between neonatal and adult erythrocytes, both blood products are currently processed and stored under identical conditions (27). Whether storage duration, additive solution composition, or processing methods differentially affect the quality and functionality of CB-RBCs compared to adult RBCs remains largely unknown and may yet prove to be a critical determinant of transfusion outcomes in this vulnerable population.

1.5 RBC storage and storage lesions

Red blood cells are typically stored at 2–6°C for up to 49 days (64,65). During this time, chemical reactions continue to take place at a reduced rate. However, physiological protective functions of the circulation are absent, and damaged cells cannot be cleared, resulting in storage lesions and cellular lysis. Storage lesions can adversely affect erythrocyte integrity and function and may have clinically relevant consequences following transfusion (66).

Since mature erythrocytes lack mitochondria, they depend entirely on glycolysis to generate ATP (45). Storage at 2–6 °C slows enzymatic activity and impairs energy-dependent ion transport, particularly Na^+/K^+-ATPase activity (67). As a result, cation homeostasis becomes progressively disturbed, with intracellular Na^+ accumulation, K^+ leakage into the storage supernatant and altered water balance. This disturbance in ion regulation is one of the earliest contributors to cell swelling, changes in cell volume and impaired membrane stability (67). At the same time, ATP and 2,3-diphosphoglycerate (2,3-DPG) are gradually depleted. The loss of 2,3-DPG increases haemoglobin oxygen affinity (68), resulting in a leftward shift of the oxygen dissociation curve and reduced oxygen release to the tissue (69). ATP depletion further compromises membrane integrity, ion pump activity and Ca^{2+} homeostasis. Increased intracellular Ca^{2+} may activate Ca^{2+}-dependent K^+ channels and proteolytic enzymes such as calpains, thereby contributing to eryptosis-like processes and reduced red cell survival (70).

Although metabolic activity is markedly slowed at low temperature, glycolysis continues during storage. Glucose is consumed, and lactate accumulates, leading to progressive acidification of the storage medium (69). While this pH decline may theoretically favour oxygen release via the Bohr effect, it also feeds back negatively on glycolytic activity and contributes to metabolic exhaustion (69). Although the drop in pH inhibits glycolytic activity, glycolysis continues, allowing glycolytic intermediates to accumulate temporarily at the start of storage – an attempt by the cell to compensate. (69). However, during prolonged storage, this compensatory capacity diminishes as oxidative modifications of glycolytic enzymes progressively increase, particularly those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (71). As a consequence, flux through late glycolysis is reduced, ATP production becomes less efficient, and 2,3-DPG is increasingly consumed to support residual ATP and lactate formation. This further aggravates both ATP and 2,3-DPG depletion (69).

A central feature of the storage lesion is the development of oxidative stress (69). Reactive oxygen species are generated mainly through haemoglobin autoxidation and iron-mediated reactions, including Fenton and Haber-Weiss chemistry, resulting in the formation of superoxide, hydrogen peroxide and highly reactive hydroxyl radicals (72). To counteract oxidative stress, glucose metabolism is partially redirected towards the pentose phosphate pathway, where NADPH is produced (66). NADPH is required to maintain glutathione in its reduced form, which is essential for detoxifying peroxides and limiting oxidative damage (67). However, this protective system becomes progressively insufficient. Reduced glutathione decreases, oxidised glutathione increases, and the uptake or retention of glutathione precursors such as glutamate, glutamine, glycine and cysteine becomes impaired (66). Thus, ATP depletion and redox imbalance reinforce one another: lower ATP availability weakens glutathione homeostasis, reduced antioxidant capacity increases oxidative stress, and oxidative stress further inhibits key glycolytic enzymes such as GAPDH (66).

These oxidative changes do not remain confined to soluble proteins and lipids but also affect key membrane components (69). Band 3, or anion exchanger 1, acts as a central regulatory hub linking metabolism, gas transport and membrane stability (73). Under physiological conditions, it coordinates oxygen-dependent metabolic modulation by dynamically binding and releasing key glycolytic enzymes (74). During storage,

oxidative modification, clustering, and fragmentation of Band 3 disrupt this regulation and weaken membrane-cytoskeleton interactions (69).

Vesiculation represents an important downstream consequence of these biochemical and structural changes. On the one hand, vesicle formation may serve as a protective mechanism by allowing the erythrocyte to remove irreversibly oxidised or functionally impaired proteins and lipids (75). On the other hand, this process causes an irreversible loss of membrane surface area (69). Storage-derived vesicles may contain haemoglobin, Band 3, phosphatidylserine-exposing membrane fragments and other damaged components. As membrane is progressively lost, the surface-area-to-volume ratio decreases, deformability is reduced and osmotic fragility increases (69). Morphologically, this contributes to the typical storage-related shift from discocytes towards echinocytes, spheroechinocytes and more spherocytic forms (75).

Ultimately, the combined effects of ATP depletion, 2,3-DPG loss, ion imbalance, oxidative damage, Band 3 dysfunction, membrane-cytoskeleton destabilisation and vesiculation increase red cell fragility and promote haemolysis (66). These processes may also impair post-transfusion recovery, as damaged or phosphatidylserine-exposing cells are more likely to be rapidly cleared from the circulation (66). Importantly, the severity of the storage lesion is not determined by storage duration alone. Donor biology, genetics, sex, age, body mass index (BMI), ethnicity, processing methods and environmental exposures can all influence how strongly individual RBC units develop metabolic, oxidative and structural storage-related damage (76). Therefore, the storage lesion should be understood not simply as a time-dependent deterioration, but as the result of interacting metabolic, redox, membrane and donor-dependent factors.

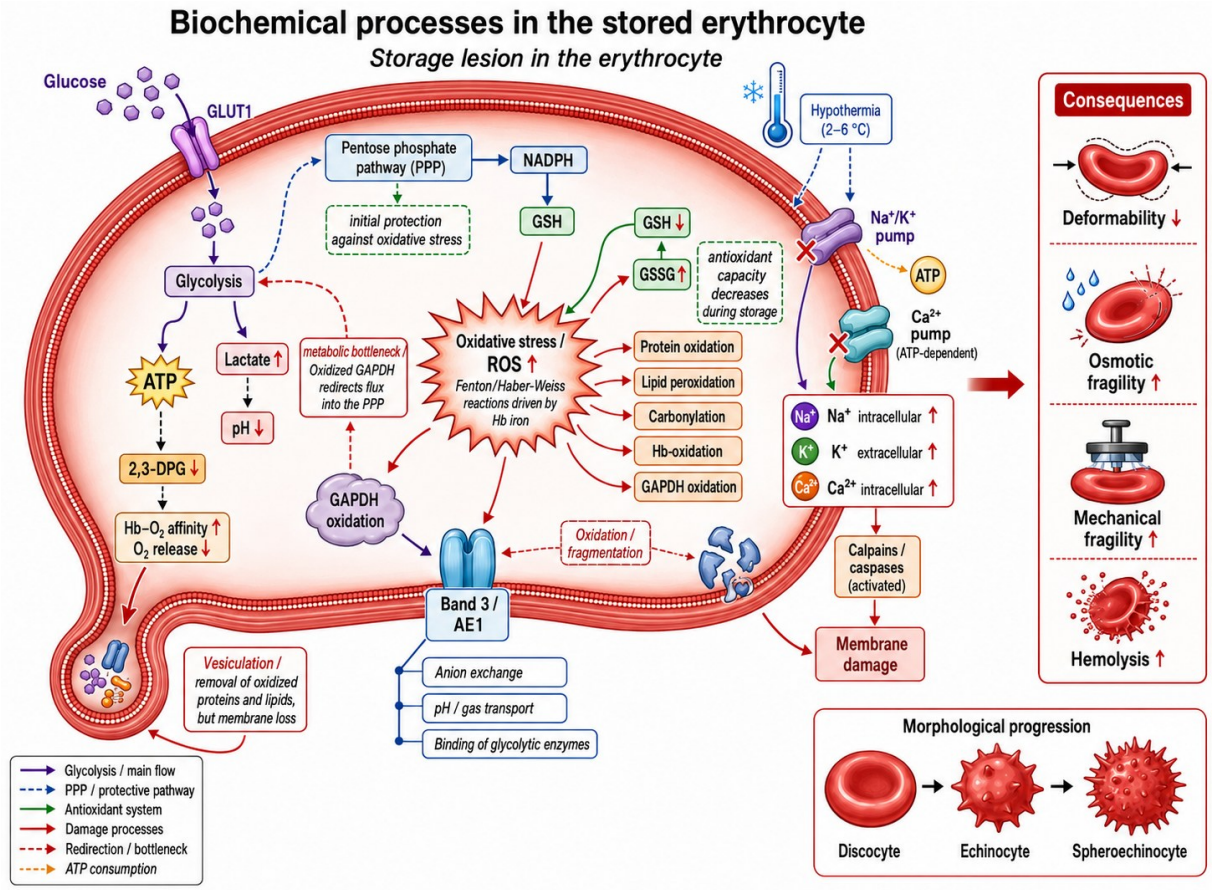

***Figure 1**: **AI-generated schematic representation of red blood cell storage lesions.** The illustration summarises major biochemical and morphological alterations associated with refrigerated erythrocyte storage at 2–6 °C, including metabolic depletion, oxidative stress, membrane and cytoskeletal damage, vesiculation, ion imbalance, phosphatidylserine exposure and loss of normal discocyte morphology. The figure was generated using artificial intelligence for visualisation purposes and is based on mechanisms described in the literature cited in the corresponding section.*

1.6 Different additive solutions for pRBC

Several different additive solutions are approved for the storage of erythrocytes. Due to the differences in composition, cells may be stored for a longer or shorter period of time. However, they may not be stored for more than 49 days (65,77). Generally, solutions differ in carbohydrate source, buffering and phosphate capacity, ionic composition, osmotic support, and the presence of membrane-stabilising or metabolism-supporting additives such as adenine, mannitol, citrate, phosphate, guanosine, and gluconate (77). The most commonly employed solutions comprise: Additive Solution 1,3,5 or 7 (AS1, AS3, AS5, AS7), Erythrocyte Additive solution 5 (E-sol 5), Saline-Adenine-Glucose-Mannitol (SAGM), Phosphate-Adenine-Glucose-Guanosine-Saline-Mannitol (PAGSM), Phosphate-Adenine-Glucose-Guanosine-Gluconate-Mannitol (PAG3M) and Mannitol-Adenine-Phosphate (MAP). The general

aim of these different additive solutions is to preserve RBC viability, minimise haemolysis, and ensure efficient oxygen delivery following transfusion. (77)

One of the most widely used additive solutions in Europe is SAGM. SAGM contains sodium chloride, adenine, glucose, and mannitol (63). The predecessor of SAGM was SAG, which already managed to decrease haematocrit and viscosity. However, cells stored in SAG showed comparatively high level of haemolysis over time (63). By adding mannitol, the rate of haemolysis decreased. Mannitol is a free radical scavenger and membrane stabiliser (63).Glucose and Adenine SAGM are responsible for supporting ATP production. NaCl is used to safeguard the osmotic stability of stored cells (63). One notable limitation of SAGM is its acidic pH of 5.7, which can contribute to protein and membrane damage over prolonged storage periods (77). Furthermore, metabolic and oxidative changes continue to accumulate during storage, and SAGM-stored red blood cells have been shown to exhibit higher osmotic fragility, increased extracellular vesicle release, and greater phosphatidylserine exposure under transfusion stress conditions. While SAGM extends the RBC shelf life to 42 days, these limitations highlight the need for further optimisation of additive solutions beyond what SAGM currently offers (77).

AS1 and AS5 are very similar to SAGM. These solution only differ in the concentrations of sodium chloride, sugar and mannitol (78). Due to their similar composition, cells stored in AS1 and AS5 also exhibit comparable biochemical behaviour to those stored in SAGM.

AS3 differs markedly from SAGM. Although it contains saline, adenine and glucose in similar concentrations, it lacks mannitol and incorporates citrate and phosphate as additional components (63). These substitutions are not merely osmotic replacements, but may confer distinct biochemical advantages. Using isotope-tracing experiments d'Alessandro et al. demonstrated that erythrocytes stored in AS3 are capable of taking up and metabolising citrate. They suggested that cytosolic Krebs cycle enzyme isoforms that are still present within the RBC cytosol may contribute to the production of reducing equivalents NADH and NADPH through pathways alternative to glycolysis and PPP , However, as this remains a mechanistic hypothesis rather than a directly demonstrated causal relationship, further studies are required (79). The inclusion of

phosphate in AS3 may support erythrocyte metabolism by providing inorganic phosphate as a substrate for glycolysis and as an allosteric activator of phosphofructokinase (PFK), thereby supporting ATP synthesis and 2,3-DPG maintenance (80,81).

The inclusion of phosphate is also a feature of AS 7, which also contains bicarbonate, adenine, glucose, mannitol, but lacks NaCl (82). AS7 was developed to support RBC metabolism by combining phosphate availability with an alkaline, bicarbonate-based buffering system (82). Its beneficial effects have been linked to the so-called chloride shift. Due to the low-chloride composition of AS 7, chloride ions diffuse out of the erythrocyte, while hydroxyl ions enter the cell to maintain electroneutrality. This results in intracellular alkalinisation, which can activate phosphofructokinase (PFK), a key regulatory enzyme of glycolysis, and thereby increase glycolytic flux (82). D'Alessandro et al. further showed that AS7 additionally promotes the PPP activity during early storage, which generates NADPH and thereby supports glutathione recycling. Total glutathione pools were 2.1-fold higher in AS7 compared to AS3 at Day 7, suggesting improved antioxidant capacity (83). Compared to AS1, AS7 stored RBCs showed significantly lower haemolysis, less microvesicle-bound protein shedding and a higher morphology index (a measure of biconcave disc shape retention, where 100 indicates a perfect biconcave morphology) throughout storage (82).

However, limitations remain. The metabolic advantages of AS7 are most pronounced during early storage and diminish over time. Furthermore, progressive depletion of the $NADP^+$/NADPH pool was observed, indicating that the redox benefit is not sustained long-term (83).

For extending and improving shelf life, next-generation solutions like PAG3M and E-Sol 5 were developed (77). PAG3M contains adenine, guanosine, gluconate, glucose, mannitol and phosphate (77). Like SAGM, it contains adenine to support ATP regeneration and mannitol as a membrane stabiliser. However, PAG3M additionally contains guanosine, which is broken down inside the erythrocyte into guanine and ribose-1-phosphate(84). Since the pentose phosphate pathway is the main source of NADPH in erythrocytes, ribose-1-phosphate derived from guanosine feeds into this pathway, increasing NADPH generation and thereby enabling glutathione reductase to

regenerate reduced glutathione (GSH) from its oxidised form (GSSG), thus supporting antioxidative capacity (85). Additionally, gluconate and an alkaline buffer promote a higher intracellular pH, which further activates glycolytic enzymes (84). This is achieved through a chloride shift: as gluconate is membrane-impermeant, it cannot replace intracellular chloride, driving a net efflux of Cl^- via the Band 3 anion exchanger coupled with an influx of OH^-, thereby raising the intracellular pH and relieving the inhibition of pH-sensitive enzymes such as phosphofructokinase, bisphosphoglycerate mutase, and glucose-6-phosphate dehydrogenase (85) . Together, these components aim to better maintain erythrocyte metabolism during storage compared to conventional additive solutions such as SAGM (84,85). A closely related solution is PAGSM, which shares the same components as PAG3M but contains saline instead of gluconate, and therefore lacks the chloride shift-promoting effect of gluconate (77).

Similarly, E-Sol 5 also constitutes a next-generation alkaline additive solution that, like PAG3M, is chloride-free and exploits the chloride shift mechanism to promote intracellular alkalinization (77). The resulting activation of glycolytic enzymes and improved redox metabolism are mechanistically comparable to those described for PAG3M. Compared to conventional solutions such as SAGM, E-Sol 5 shows better preservation of ATP and 2,3-DPG, lower haemolysis, and improved morphology scores throughout storage (86).

Another commonly used additive solution is MAP, which contains sodium chloride, glucose, mannitol, adenine, citrate and phosphate (77). Each of these constituents serves a specific role in preserving RBC function during storage: glucose provides substrate for glycolysis, adenine supports ATP synthesis, and phosphate acts as a buffer. Adequate Mannitol, in turn, is thought to act as an osmotic stabiliser, thereby protecting erythrocytes from osmotic stress-induced lysis during storage (77).

The usage of an additive solution usually depends on national and international standards. SAGM is mainly used in Europe (87), the UK (67) and Australia (88). AS1 and AS5 are used in the United States, whereas the usage of AS3 has also extended to Canada. (87) Even though SAGM is the leading solution throughout most of Europe, PAGSM is frequently employed in Germany. In Japan, MAP is the primary solution

used for storing erythrocytes. (89) Until now, PAG3M and E-Sol 5 have only been used for experimental settings (77).

Constituente (mmol/L)	AS1	AS3	AS5	AS7	E-Sol 5	SAGM	PAGSM	PAG3M	MAP
NaCl	154	70	150	–	–	150	72	–	85
$NaHCO_3$	–	–	–	26	–	–	–	–	–
Na_2HPO_4	–	–	–	12	20	–	16	8	–
NaH_2PO_4	–	23	–	–	–	–	8	8	6
Gluconate	–	–	–	–	–	–	–	40	–
Citrate	–	2	–	–	25	–	–	–	1
Na-Citrate	–	23	–	–	–	–	–	–	5
Adenine	2	2	2.2	2	2	1.25	1.4	1.4	1.5
Guanosine	–	–	–	–	–	–	1.4	1.4	–
Glucose	111	55	45	80	111	45	47	47	40
Mannitol	41	–	45.5	55	41	30	55	55	80
pH	5.5	5.8	5.5	8.5	8.4	5.7	8.2	8.2	5.7

Table 2: *Composition and pH of most used additive solutions to store red blood cells.*

1.7 Aim of the thesis

Preterm infants often require red blood cell transfusions, yet adult erythrocytes differ fundamentally from neonatal erythrocytes in structure, metabolism and oxygen transport. These differences may also affect how both cell types respond to hypothermic storage and the chemical composition of additive solutions.

Therefore, the aim of this thesis is to systematically compare adult and neonatal erythrocytes during storage in different additive solutions by assessing haemolysis, cell count, morphological alterations, glucose utilisation and lactate accumulation over time. This work aims to identify whether neonatal erythrocytes show distinct storage characteristics that could be relevant for transfusion strategies in preterm infants and improve blood banking and allocation throughout the clinical implementation of the novel blood product.

2 Materials and Methods

Table 3: Chemicals and reagents used in this study.

Substance / Reagent	Company	Product number
tri-Natriumcitrat-Dihydrat	Merck	1064461000
D-Mannitol	Merck	1371621000
Adenin	Merck	A2786-25G
Guanosin	Merck	G6264-25G
Sodium chloride (NaCl)	Merck	1064041000
Disodium hydrogen phosphate dihydrate	Merck	1065761000
Sodium dihydrogen phosphate dihydrate	Merck	1370181000
Sodium hydrogen carbonate	Merck	S5761-500G
Citric acid	Merck	27487-250G-F
Dextrose (Glucose)	Merck	1370481000
Sodium hydroxide (NaOH)	Merck	1370321002
Hydrochloric acid (HCl)	Merck	1003141000
Glucose Hexokinase FS assay kit	DiaSys Diagnostics	—
Lactate FS assay kit	DiaSys Diagnostics	—

Table 4: Consumables used in this study

Material	Manufacturer	Product number
96-Well Masterblock, 2 mL, Polypropylen	Greiner Bio-One	780271
Axygen® Universal Fit Filter Pipette Tips	Corning	TF-1005-WB-R-S
Falcon® Conical Centrifuge Tubes (50 mL)	Corning	352070
Falcon® Conical Centrifuge Tubes (15 mL)	Corning	352096
Omnifix® 10 mL Spritze, Luer-Ansatz (exzentrisch)	B. Braun	4616103V
Blunt Fill Needle (1.2mm x 40mm)	BD	303129
Nunc™ Seals	Thermo Scientific	232698

2.1 Additive solutions and Setup

Seven different EMA (European Medicine Agency) or FDA (Food and Drug Administration) approved additive solutions were prepared in-house: SAGM, AS1, AS3, AS5, AS7, PAGSM and PAG3M. The chemical composition of each additive solution is listed in table 2. Only fresh pharmaceutical grade chemicals were used as components, and solutions were sterile filtered using a Nalgene™ Rapid-Flow™ Sterile Disposable Filter Unit prior to usage. For poorly soluble additive solutions, such as the guanosine-rich PAGSM and PAG3M, care was taken not to heat chemicals to temperatures above 50°C to avoid decomposition. To verify the veracity of the preparations, pH-values and osmotic pressure of the solutions were determined experimentally. In case of an osmotic discrepancy larger than 5% from the calculated value, the respective solution was prepared anew.

For longitudinal storage experiments, a previously validated 96-deep-well plate workflow was used for high-throughput screening of different additive solutions (90).

Compared with conventional PVC blood bag storage, this miniaturised platform requires substantially fewer resources while enabling simultaneous testing of multiple storage conditions. Nikulina et al. reported that key storage lesion parameters, including haemolysis, ATP depletion, and metabolic alterations, were comparable between deep-well plate and conventional storage systems, validating its use as a preliminary screening platform (90).

Finally, each well plate was sealed air-tight using a sterile Nunc™ aluminium sealing tape. Samples were stored at 4 °C to mimic standard blood bank storage conditions, which is within the recommended storage temperature range of 2–6 °C for erythrocyte concentrates according to European Commission Directive 2004/33/EC (91).

2.2 Sample Collection and Processing

Informed consent was obtained prior to any invasive procedure (EK2040/2022). 36mL of venous blood was drawn directly after caesarean section from a proximal portion of the umbilical cord into CPD-A (citrate-phosphate-dextrose-adenine) tubes. Visibly damaged umbilical cords and samples with unstable blood flow into the evacuated CPD-A tubes were excluded from further processing. Only healthy, term-born infants with a birthweight between 2500g and 4000g were included in the study. Exclusion criteria were pregnancy-associated disease of the mother, maternal infectious diseases, laboratory signs of inflammation 24h prior to birth, as well as neonatal congenital malformations and chromosomal aberrations.

To allow for better comparability between cord blood and adult red blood cells, peripheral blood was obtained from healthy adult volunteers within 15 minutes of cord blood collection. Volunteers were aged between 25 and 28 years, with a weight between 58 and 87 kg and a height between 160 and 189 cm. Irrespective of the sample origin, tubes were immediately placed at 4°C and processed immediately thereafter.

2.3 RBC Isolation

In order to enable a comparative investigation of the storability of CB and adult-derived erythrocytes in different additive solutions, red blood cells were isolated from whole blood by removing leukocytes and platelets.

To establish an optimised isolation protocol, preliminary experiments were performed using adult whole blood. These experiments were based on an isolation protocol used in previous studies and included the evaluation of different anticoagulants, storage platform types, and sample volumes. Once optimal conditions were identified, the modified protocol was applied in subsequent screening experiments.

All procedures were performed under sterile conditions in a laminar-flow biosafety cabinet using sterile equipment unless otherwise stated.

2.3.1 Preliminary RBC Isolation Protocol

CPD-A anticoagulated whole blood was gently inverted several times and 12 mL were transferred into a sterile 15 mL Falcon tube. The remaining fraction of blood was stored at 4°C for later analysis of cell composition. The transferred sample was centrifuged at 280 g for 8 min at 4°C without breaks, a procedure previously used to prepare platelet-rich plasma from umbilical cord blood (92) . The platelet rich fraction was discarded, and the sample was equivoluminally resuspended in PBS and centrifuged using the same settings. The supernatant (SN) was discarded again and the platelet-depleted, leukocyte-rich erythrocyte pellet was resuspended in PBS. The suspension was aliquoted into 2 mL tubes, and centrifuged at 10 000 g for 2 min. Following centrifugation, the buffy coat and supernatant were removed, the remaining cells were pooled into a 15 mL sterile Falcon tube and the volume was adjusted to 12 mL using PBS. The suspension was then passed through an Arcodisc™ WBC (white blood cell) syringe filter, and the leukodepleted RBC sample pooled in a sterile 50 mL Falcon. To deplete remaining plasma, the filtrate was centrifuged at 3200 g for 15 min at 4°C without breaks. The main fraction of the SN was then aspirated using an automated suction pump and residual SN carefully removed with a 100 µl pipette to reduce variability in cellular concentration between processed blood samples. Prior to resuspension in additive solution, the pRBC (packed red blood cell) sample was gently

vortexed and carefully resuspended manually using a 1000 µL pipette to avoid cell density bias when adding packed cells from the top-or bottom fraction of the tube into the 96-deep well plate.

2.3.2 Optimisation of the Preliminary Protocol

Building on the existing storage protocol, it was systematically evaluated whether the proposed blood collection tubes (CPD-A) and storage platform (96 deep-well plate) provide favourable storage conditions.

To assess the suitability of different storage platforms, 600 µL of freshly isolated red blood cells were mixed with 400 µL of storage additive solution (SAGM, AS1, AS3) and aliquoted into either 2 mL microcentrifuge tubes, 15 mL Falcon tubes, or 96 deep-well plates sealed with autoclaved Nunc™ aluminium sealing tape. All samples were stored at 4 °C for a total of four weeks.

To investigate potential volume-dependent effects on storage performance, erythrocytes were prepared as described above and stored in volumes of 300 µL, 600 µL, and 1000 µL, maintaining a 3:2 ratio of packed red blood cells to additive solution. Samples were aliquoted into 96 deep-well plates, sealed with aluminium tape, and stored at 4 °C.

In addition, experiments were repeated using CPD-A and heparin tubes as whole blood collection devices to compare their impact on storage quality. Peripheral blood from adult donors was processed and stored as described above.

To reduce the haemolysis caused by samples processing, red blood cells may be washed once after 24h of storage. Tests were carried out to determine whether it is better to perform the washing step once or twice, and whether washing should take place after 24 hours or after 48 hours.

Furthermore, a test was carried out to demonstrate that cells stored in solutions that were produced in-house show similar characteristics as cells stored in GMP-grade solutions. To this end, SAGM produced in-house was compared to SAGM provided by

the Austrian Red Cross. The samples were prepared as described above, and haemolysis was measured once a week over a four-week period.

2.3.3 Optimised Isolation Method

Isolation of red blood cells using the newly established protocol was performed from CPD-A anticoagulated whole blood. Briefly, 14mL of blood was carefully poured into a 15 mL Falcon tube, and the sample was centrifuged at 3200 g, 4°C, for 15 min without brakes. After centrifugation, the tip of the Falcon tube was briefly immersed in ethanol, the tube was fixated on a self-3D-printed PVC-mount, and the tip allowed to dry for a few minutes. The bottom end of the tube was then punctured with a sterile blunt fill needle (1.2mm x 40mm), and the cellular fraction of the blood collected into a fresh 15 mL Falcon tube. The remaining sample, including the buffy coat and plasma, was discarded. The collected red blood cell fraction was adjusted to a final volume of 12 mL in PBS and passed through an Acrodisc™WBC (white blood cell) syringe filter. The platelet-poor, leukocyte-depleted erythrocytes were then resuspended to a final volume of 12mL in PBS, centrifuged under the same conditions as before (3200 g, 4°C, 15 min, no breaks), and the supernatant was carefully removed. Derived pRBCs were then used for subsequent storage experiments.

2.3.4 Sysmex

To verify the success and quality of pRBC isolation, samples were analysed using a Sysmex XN-350 haematology analyser. 475 µL of PBS was transferred into a 1.5 mL microcentrifuge tube, followed by the addition of 25 µL of packed erythrocytes taken from the remaining fraction of pRBCs not transferred into the 96-deep-well storage platform.

Additionally, a small aliquot of WB preserved in CPD-A tubes at 4°C was used for reference measurements. Briefly, 1.5 mL of CPD-A anticoagulated whole blood was centrifuged at 10,000 g for 2 min. Plasma was removed without disturbing the buffy coat. Cells were homogenised and diluted 1:20 in PBS prior to measurement.

2.4 Downstream Processing of Isolated Erythrocytes

For storage experiments, 560 µL of different additive solutions were dispensed into a 96-deep-well plate. Subsequently, 840 µL of packed erythrocytes were added, using a wide-bore pipette tip, manually mixed 5 times to ensure homogeneity and the plates were sealed with a sterile Nunc™ aluminium sealing tape. Each plate contained two to four different additive solutions for cord blood and adult blood, respectively. As two plates were used per experimental batch, between five and seven solutions were analysed per experiment. The plates were stored in a styrofoam box in a cold room between 2°C and 6°C.

To account for processing-induced lysis of fragile cells, a washing step was performed on each sample after 24 hours of storage. Briefly, red blood cells and additive solution were manually resuspended five times by pipetting with a wide-bore tip prior to transfer into 2 mL microcentrifuge tubes. The suspensions were centrifuged at 500 g, 4°C for 10 min, and 100µL of SN were collected for the determination of haemolysis. The remaining SN was discarded without disturbing the pRBC pellet. The cells were resuspended equivoluminally in the respective additive solution using a wide-bore pipette tip. Afterwards, samples were transferred into a 96-deep well plate, the plate was sealed, and storage continued at 4°C in a cold room.

2.5 Storage Screening

To assess the storability of neonatal and adult erythrocytes in different additive solutions over four weeks, samples were collected weekly from the deep-well plate and examined.

Each sample was slowly mixed five times under sterile conditions using a wide-bore pipette tip. Afterwards, 180 µL of sample were transferred into a fresh microcentrifuge tube. Special care was taken to immediately reseal plates and continue storage at 4°C after retrieving the samples.

2.5.1 Light Microscopy

90 µL fixation solution (formaldehyde 16%, glutaraldehyde 50%, PBS) was prelayered into 1.5 mL microcentrifuge tubes, and 10 µL of red blood cell suspension, obtained directly from the stored plate, slowly added. The tubes were mixed three times, placed in a 50 mL Falcon tube padded with cellulose (to avoid unnecessary shear forces), and spun on a roller mixer at moderate speed for 30 min. After incubation, cells were resuspended in 1 mL PBS and centrifuged at 500g, 4°C for 10 min. The SN was discarded, and the cell pellet resuspended in 100 µL PBS. The fixed samples were stored at 4°C for a maximum of seven days prior to usage for blood smears. For blood smears, samples were resuspended, and 10 µL of the suspension was smeared onto a microscope slide like a blood film and air-dried. After drying, samples were fixed for 30 seconds with Methanol, air-dried and covered with Eukitt. Stained samples were automatically coloured following the Wright with Hematek 2000® method, air-dried and covered with Eukitt.

Morphological analysis was performed by classifying erythrocytes into three subclasses: (i) discocytes + stomatocytes, (ii) echinocytes + echinospherocytes, and (iii) spherocytes. Parameters at week four were analysed separately for cord blood and adult erythrocytes using a REML-based mixed effects model with additive solution as the fixed factor. A minimum of 300 cells was counted per sample. Reproducibility was assessed by analysing additional fields of view (n = 15), confirming a reproducibility of ±1.5%. A test set of 16 PRBC samples was independently evaluated by two observers, showing an inter-observer variability of less than ±2%, confirming robustness of the counting method. Graphs for lactate and glucose were generated using R (version 4.5.2).

2.5.2 Imaging Flow

9µL from the freshly obtained pRBC suspension were resuspended in 1791µL PBS and stored for a maximum of 2h at 4°C prior to measurement on the Amnis Imagestream MK-II imaging flow cytometer. 50.000 brightfield images were recorded for each sample using high sensitivity settings in conjunction with 60x magnification

and a core size setting of 6µm. Event rates were kept constant to achieve optimal resolution.

2.5.3 Haemolysis

pRBCs were centrifuged at 10.000 g, 2 min, at 4°C. The supernatant was transferred to a new microcentrifuge tube and recentrifuged using the same settings. The remaining cells were kept on ice until further analysis. After the second centrifugation step, 55 µL of supernatant was transferred to a new tube and stored at -20°C until measurement. For quantification of haemolysis, samples were thawed at room temperature. The samples were diluted 1:6 in in-house-made Drabkin's Reagent (11.9 mM $NaHCO_3$, 0.77 mM KCN and 0.61 mM $K_3[Fe(CN)_6]$ in ddH_2O.) then incubated for 30 min at RT prior to measurement at 540 nm. Samples collected at timepoints between 24 h and three weeks were measured in triplicate; samples collected at the final timepoint of four weeks were measured in sextuplicate.

To account for differences in sample handling as well as cell density between samples, 100 µL of pRBC suspension were collected at week four of storage and placed at -80°C. Samples were then homogenised by three cycles of freeze-thaw using LN_2 (liquid nitrogen), diluted 1:60 in ddH2O and haemolysis was measured in sextuplicate using Drabkin's Reagent on two different experimental days. Consequently, haemolysis values obtained from stored samples were adjusted for cell density differences between samples by dividing the haemolysis values by the sample-specific normalisation factor derived from the corresponding standardisation samples.

2.5.4 Metabolomics

The cells that had been stored on ice during recovery of the SN for the quantification of haemolysis were centrifuged again at 10.000 g for 2 min at 4°C. Any remaining SN, if still visible, was removed and discarded. The cells were then snap-frozen in LN_2 and stored at -80°C until measurement.

2.5.5 Measurement of Glycolytic Activity

2.5.5.1 Precipitation of Samples in Trichloroacetic Acid

Red blood cell pellets stored at -80°C were thawed on ice. Cells were homogenised, and 20µL of suspension/hemolysate was diluted in 50µL ddH_2O. 130µL of 10% trichloroacetic acid (TCA) was added to the hemolysate to give a final RBC dilution of 1:10, and the samples were vortexed at full speed for 20 s, followed by pulse vortexing. The samples were then incubated on ice for 10 min, followed by centrifugation at 20.000 g at 4°C for 10 min. 130 µL of precipitation SN were transferred into a fresh microcentrifuge tube and placed on dry ice prior to transfer to -80°C.

Precipitation controls were generated by mixing 50µL ddH_2O with 10µL of 400 mM glucose, 120µL of 10% TCA and 20µL of the respective sample.Lactate

Lactate measurements were performed using the Lactate FS kit from Diagnostic Systems with all reagents equilibrated to 37 °C prior to use. Due to foam formation in reagent R1, the assay was not conducted in a 96-well plate format. Instead, empty 1.5 mL microcentrifuge tubes were pre-warmed to 37 °C. Subsequently, 1000 µL of reagent R1 was added, followed by 15 to 30 µL (depending on storage timepoint) of the precipitation SN. The mixture was incubated for 5 min at 37 °C on a thermoshaker at 600 rpm. Thereafter, the reaction mixture was transferred into a cuvette and absorbance was measured at 340 nm with a Hitachi U-2000 spectrophotometer. . Next, 677 µL of the cuvette content was transferred into a fresh pre-warmed microcentrifuge tube, and 167 µL of reagent R2 (37 °C) were added. After incubation for 5 min at 37 °C at 600 rpm, the reaction mixture was transferred into a new cuvette and absorbance was measured at 340 nm. For each kit in use and sample volume added to reagent R1, standard-curves were prepared and values extrapolated to absolute concentrations.

2.5.5.2 Glucose

Glucose measurements were performed using the Glucose Hexokinase FS kit, from Diagnostic Systems. Prior to analysis, all reagents were equilibrated to room temperature. Subsequently, 200 µL of reagent R1 was added to each well of a 96-well plate, followed by the addition of 10 µL of the precipitation SN. After thorough mixing,

samples were incubated for 2 min at RT, and absorbance was measured at 340 nm using a VICTOR Nivo Multimode Microplate Reader. Thereafter, 50 µL of reagent R2 were added to each well, followed by an additional mixing step. Samples were incubated for 10 min at room temperature before a second absorbance measurement was performed at 340 nm. A standard curve ranging from 10 mM to 0.15625 mM was prepared for quantification and values extrapolated to absolute concentrations.

2.6 Statistical analysis

Data analysis and visualisation were performed using GraphPad Prism (version 11.0.1), R (version 4.5.2), and Microsoft Excel. Data are presented as mean ± standard deviation (SD). For the comparative screening of ontogeny- and additive solution-dependent effects on red blood cell haemolysis during storage, six independent samples were analysed for each storage solution–ontogeny combination, except for SAGM-stored samples, for which seven independent donors were included per ontogeny.

A two-way repeated-measures ANOVA was used to assess the effects of storage time and storage platform on haemolysis. Pairwise comparisons following significant main effects or interactions were performed using Tukey's multiple comparisons test, with $p < 0.05$ considered statistically significant.

To accommodate missing observations and the resulting unbalanced dataset, REML-based mixed-effects models were used to investigate the effect of storage volume on haemolysis over time. Storage volume and storage time were included as fixed effects, while donor was included as a random effect. Post hoc comparisons following significant main effects or interactions were performed using Tukey's multiple comparisons test, with $p < 0.05$ considered statistically significant.

REML-based mixed-effects models were similarly used to analyse isolation efficacy, with isolation protocol (old vs. new) and storage time included as fixed effects and donor included as a random effect. Post hoc comparisons were performed using Šídák's multiple comparisons test. The effects of ontogeny and storage platform on

conventional haematological parameters were analysed using the same statistical approach with Tukey's multiple comparisons test.

Pairwise t-tests were performed to analyse platelet and leukocyte depletion efficacy; no post hoc correction was applied as each parameter represented a single comparison.

Baseline haemolysis prior to washing (week 0) was analysed using a two-way ANOVA with additive solution and ontogenetic group as fixed factors, followed by Šídák's multiple comparisons test, with $p < 0.05$ considered statistically significant. Haemolysis at week 4 was analysed using a REML-based mixed-effects model with additive solution and storage time included as fixed effects and donor included as a random effect, followed by Tukey's multiple comparisons test. Changes in haemolysis over the storage period were analysed using a REML-based mixed-effects model with additive solution and storage time included as fixed effects and donor included as a random effect. Tukey's multiple comparisons test was applied following significant main effects or interactions. Specific additive solutions were omitted from individual experimental runs due to limited sample availability in order to ensure a minimum of six independent observations per additive solution. Cord blood and adult erythrocytes were analysed separately.

As the experimental design involved repeated measurements within donors, REML-based mixed-effects models with donor included as a random effect were also used to analyse changes in glucose and lactate concentrations over time. Tukey's multiple comparisons test was applied following significant main effects or interactions.

Morphological assessment included both descriptive evaluation and quantitative analysis. Spherocyte proportions were compared between cord blood and adult erythrocytes across additive solutions using a REML-based mixed-effects model with additive solution and storage time included as fixed effects and donor included as a random effect, followed by Tukey's multiple comparisons test, with $p < 0.05$ considered statistically significant.

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3 Results

3.1 Optimisation of the experimental workflow

3.1.1 Selection of collection tubes and anticoagulant

To determine the most suitable collection tube and anticoagulant for subsequent experiments, CPD-A and lithium heparin-based tubes were compared regarding storage-induced haemolysis. Testing was performed across two additive solutions (SAGM, AS1) and two storage platforms (deep well plate and 1 mL microcentrifuge tube) to ensure consistent observations across experimental conditions. Regardless of additive solution and storage platform, haemolysis levels were comparable between both anticoagulant conditions. After four weeks of storage, haemolysis in CPD-A tubes reached 2.6% (mean, n = 4 technical replicates, single donor), compared to 2.3% in lithium-heparin tubes (Figure 2). Due to the exploratory nature of this preliminary experiment, no formal statistical analysis was performed. Since CPD- based anticoagulants represent the standard in European blood collection systems (93), these tubes were selected for all subsequent experiments.

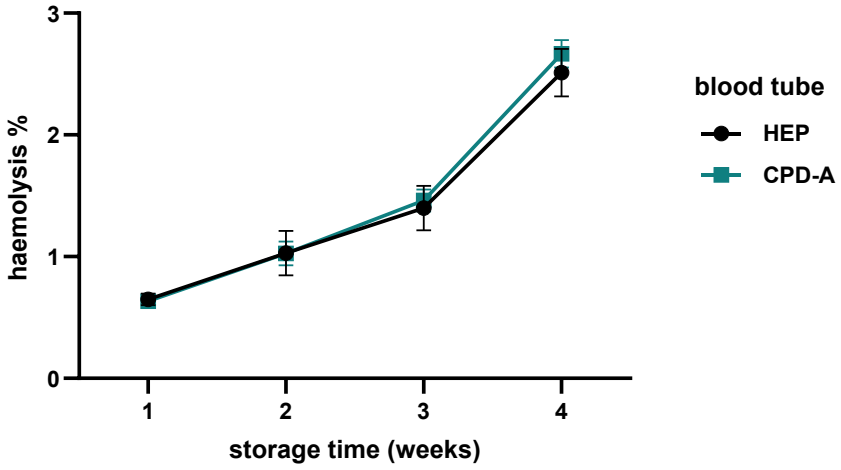

Figure 2: *Haemolysis (%) of cold-stored erythrocytes collected in CPD-A and lithium-heparin tubes at a volume of 800 µL over the course of four weeks. Data are presented as mean ± SD (n = 4, technical replicates), representing measurements obtained from two additive solutions (SAGM, AS1) across two different storage platforms (deep well, microcentrifuge tube).*

3.1.2 Evaluation of the storage platform

To identify a suitable storage platform for longitudinal experiments, deep-well plates, 15 mL Falcon tubes, and 2 mL microcentrifuge tubes were evaluated regarding storage-induced haemolysis of adult red blood cells over four weeks. Each storage platform was filled with 1 mL of a 60% erythrocyte suspension in SAGM.

Haemolysis increased over time in all storage platforms. However, erythrocytes stored in 15 mL Falcon tubes consistently showed the highest haemolysis levels throughout the storage period, whereas deep-well plates exhibited the lowest haemolysis levels. After four weeks of storage, haemolysis in erythrocytes stored in deep-well plates reached 2.56%, compared to 3.08% in 2 mL microcentrifuge tubes and 3.39% in 15 mL Falcon tubes (Figure 3). Two-way repeated measures ANOVA showed a significant effect of storage platform on haemolysis (p-value = 0.0015). However, post-hoc comparison (Tukey's multiple comparisons test) between storage platforms within individual timepoints did not reveal significant pairwise differences. Due to their lower haemolysis levels, deep-well plates were selected for subsequent experiments.

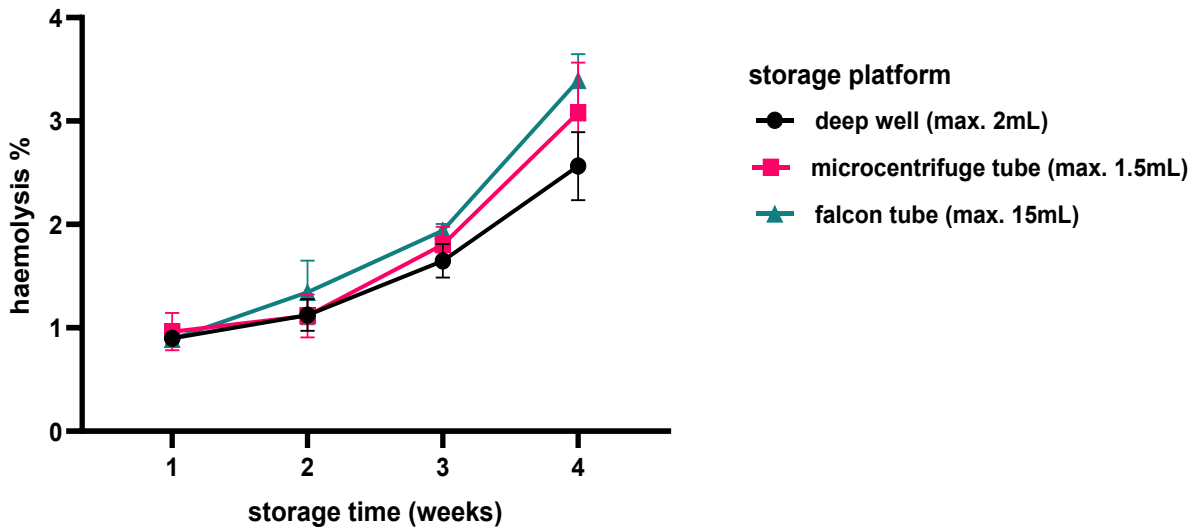

Figure 3: *Haemolysis (%) of adult erythrocytes cold stored using different storage platforms (deep well plate, 1.5 mL microcentrifuge tube and 15 mL Falcon tube) at a volume of 1 mL over the course of four weeks. Values are presented as mean ± SD (n = 3).*

3.1.3 Optimisation of storage volume

To evaluate the impact of storage volume on erythrocyte stability, adult samples were stored at volumes of 300, 600, and 1000 µL and monitored for haemolysis over time. Haemolysis decreased with increasing storage volume, indicating improved erythrocyte stability at higher sample volumes (Figure 4). During the first week of storage, haemolysis levels were comparable across all tested volumes. However, differences became apparent from week two onwards, with haemolysis increasing as storage volume decreased. At week two, haemolysis was highest in samples stored at 300 µL (mean = 1.45%, n = 3), compared to 600 µL (mean = 1.13%, n = 3) and 1000 µL (mean = 0.85%, n = 3). Using REML mixed effects model, a significant main effect of storage volume on haemolysis was observed (adj. p-value = 0.017), with a significant interaction between volume and storage time (adj. p-value = 0.048), indicating that haemolysis increased at different rates depending on the storage volume. Post hoc analysis (Tukey's multiple comparison) of pairwise comparisons revealed that differences between volumes emerged over time. At week two, haemolysis was significantly lower in the 1000 µL group compared to 300 µL (adj. p-value = 0.034), and at week three, the 1000 µL group remained significantly lower than the 600 µL group (adj. p-value = 0.050). Due to repeated sampling, the 300 µL condition could not be analysed beyond this time point. Similarly, samples stored at 600 µL were depleted after week three. In contrast, samples stored at 1000 µL remained stable throughout the entire four-week storage period and consistently showed the lowest haemolysis levels, reaching a mean of 1.95% (n = 3) after four weeks.

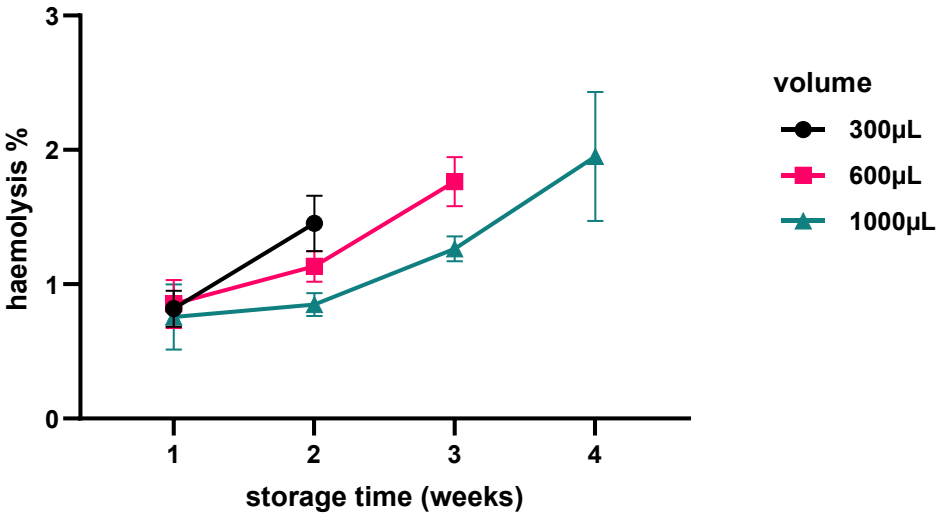

Figure 4: *Haemolysis (%) of cold-stored erythrocytes in deep well plates at different storage volumes (300, 600 and 1000 µL) over four weeks of storage. Data are presented as mean ± SD (n = 3).*

3.1.4 Final experimental setup

Since only one erythrocyte suspension could be prepared per donor at a time, a fixed starting volume applicable across all experimental conditions was required to ensure comparability. Based on the finding that higher storage volumes were associated with lower haemolysis, the highest feasible volume was selected. This was constrained by two practical considerations- Firstly, the need for four weekly withdrawals of 180 µL throughout the storage period, and secondly the requirement not to overfill the deep-well plate, which would compromise adequate mixing and handling during sample preparation. A starting volume of 1.400 µL was therefore chosen as a pragmatic compromise, ensuring a residual volume of approximately 680 µL at week 3 while maintaining sufficient sample for continued analyses.

The optimised isolation method, as described in the Materials and Methods section, involved puncturing a Falcon tube with a blunt fill needle for cell extraction after centrifugation rather than manual extraction with a pipette, coupled to several intermittent centrifugation steps. To confirm that the revised isolation approach yielded a sufficiently pure erythrocyte suspension, leukocyte and platelet counts were assessed in adult and neonatal samples. The optimised isolation technique effectively reduced both platelet and leukocyte count in all tested sample types, with a highly

significant reduction compared to whole blood in both adult and cord blood samples, as determined by a paired t-test (p-value < 0.0001) (Figure 5).

To determine whether the revised isolation approach additionally reduced cellular stress, storage-induced haemolysis was assessed in adult samples stored in SAGM, AS1, or AS5 and processed using either the previous or revised isolation methodology (Figure 6). The optimised isolation technique resulted in lower haemolysis levels across all three additive solutions. In SAGM, haemolysis at week four decreased from 3.49% (previous isolation, n = 4) to 1.26% (optimised isolation, n = 4). Similarly, week four haemolysis was reduced in AS1 from 2.93% (n = 4) to 1.11% (n = 4), and in AS5 from 3.23% (n = 3) to 0.881 % (n = 3). A REML mixed effects model revealed a significant effect of the isolation technique on haemolysis (p-value = 0.0002), with post-hoc comparison (Šidák's multiple comparisons test) confirming significantly lower haemolysis with the optimised technique for all three additive solutions (SAGM: adj. p-value = 0.026, AS1: adj. p-value = 0.001, AS5: adj. p-value = 0.037).

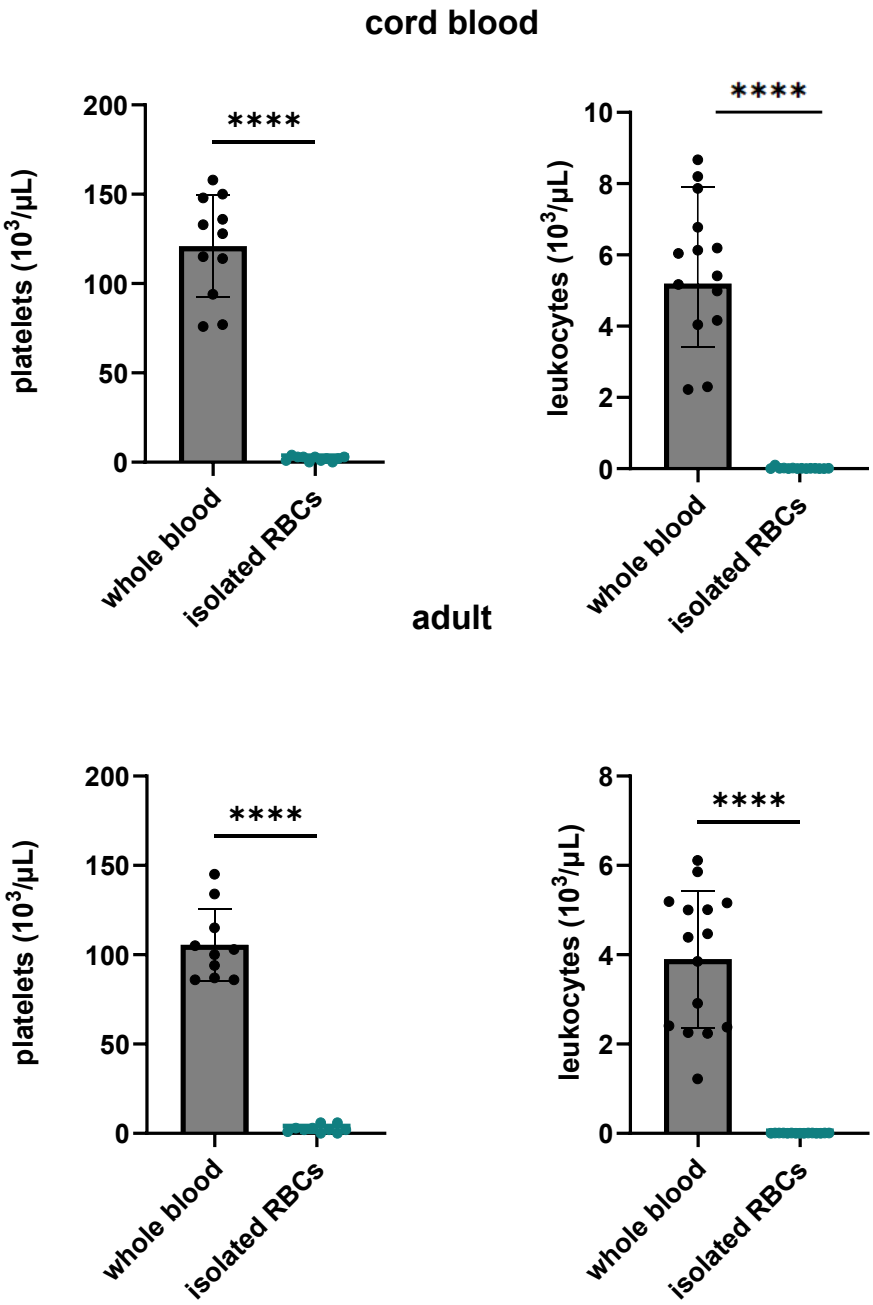

***Figure 5**: Comparison of platelet and leukocyte counts between whole blood and isolated RBC samples following erythrocyte isolation. Platelet counts and leukocyte counts are shown for cord blood (upper panels) and adult (lower panels). A marked reduction in both platelet and leukocyte count was observed after isolation. Data are presented as mean ± SD with individual data points shown. Statistical significance is indicated as *p.adj < 0.05; **p.adj < 0.01; ***p.adj < 0.001; ****p.adj < 0.0001.*

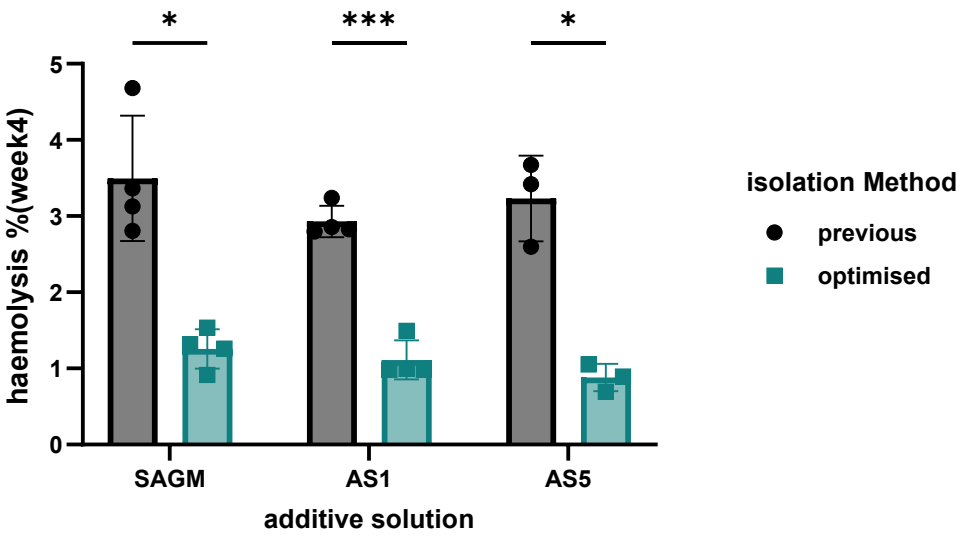

Figure 6: *Comparison of week 4 haemolysis (%) in erythrocytes isolated using the previous and the optimised isolation method across three different additive solutions. Data are shown as mean ± SD (n = 4). Statistical significance is indicated as *p.adj < 0.05; **p.adj < 0.01; ***p.adj < 0.001; ****p.adj < 0.0001.*

3.2 Initial haematological characterisation after washing of cells

Following 24 h of refrigerated storage and subsequent washing, the mean corpuscular volume (MCV), red cell distribution width (RDW-CV) and mean corpuscular haemoglobin concentration (MCHC) of adult and cord blood erythrocytes was analysed on a hemacytometer to assess differences in cell volume, size heterogeneity and haemoglobin concentration. In cord blood cells, additive solutions performed similarly, with only one significant difference observed (AS3 vs. AS7, adj. p = 0.008). In adult cells, a more pronounced solution-dependent effect was evident, with AS1 and AS7 differing significantly from most other solutions, suggesting these two solutions deviate most from the overall group behaviour (REML mixed effects model with Tukey's multiple comparissons test). Across all tested additive solutions, cord blood erythrocytes generally showed significant higher MCV values than adult erythrocytes (adj. p-value < 0.0001), indicating a larger cell volume (Figure 7), as tested using a mixed effects model. In contrast, MCHC values were largely comparable between both groups, with only minor solution-dependent variation (Figure 8). These differences were not statistically significant. RDW-CV was significantly higher in cord blood erythrocytes (adj. p-value = 0.0001) (Figure 9), suggesting greater heterogeneity in erythrocyte size compared with adult cells. Post hoc analysis showed significance in

all storage solutions comparing adult cells with cord blood cells. Overall, these findings indicate that, after preparation, cord blood and adult erythrocytes differed mainly in cell volume and size distribution, whereas cellular haemoglobin concentration was less distinctly affected by cell type.

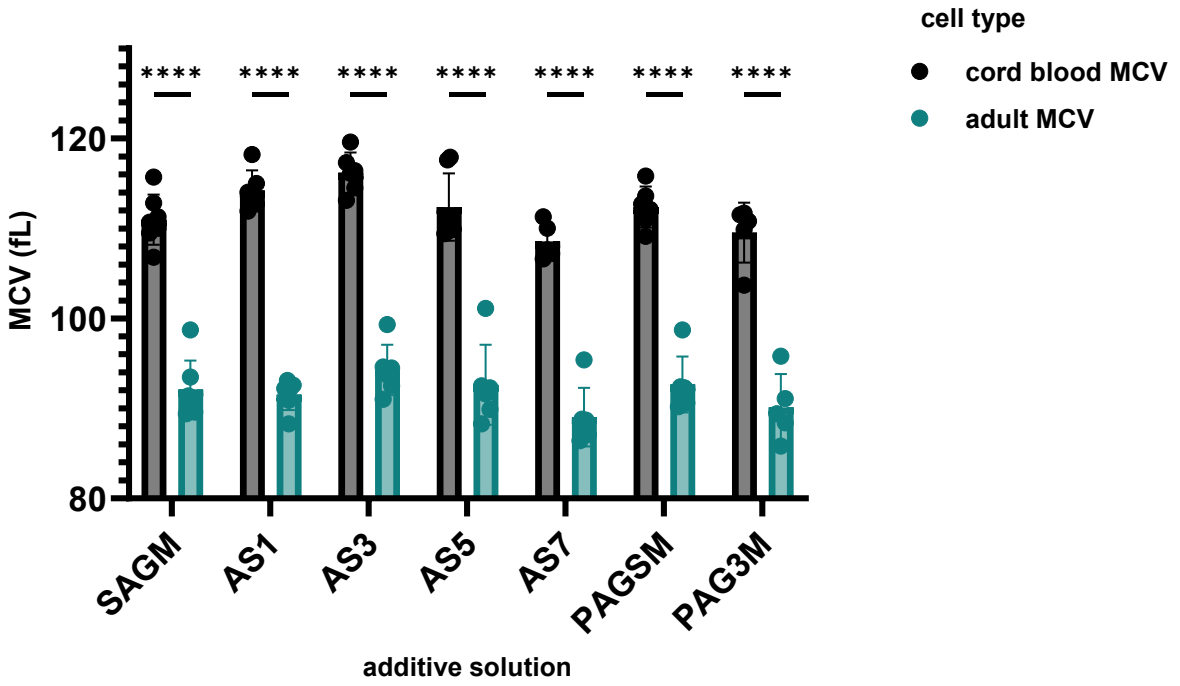

***Figure 7**: MCV (fL) of adult and cord blood erythrocytes after 24 h refrigerated storage and washing. MCV was assessed across the different additive solutions. Data are shown as individual values with mean ± SD, (n = 7). Statistical significance is indicated as *p.adj < 0.05; **p.adj < 0.01; ***p.adj < 0.001; ****p.adj < 0.0001.*

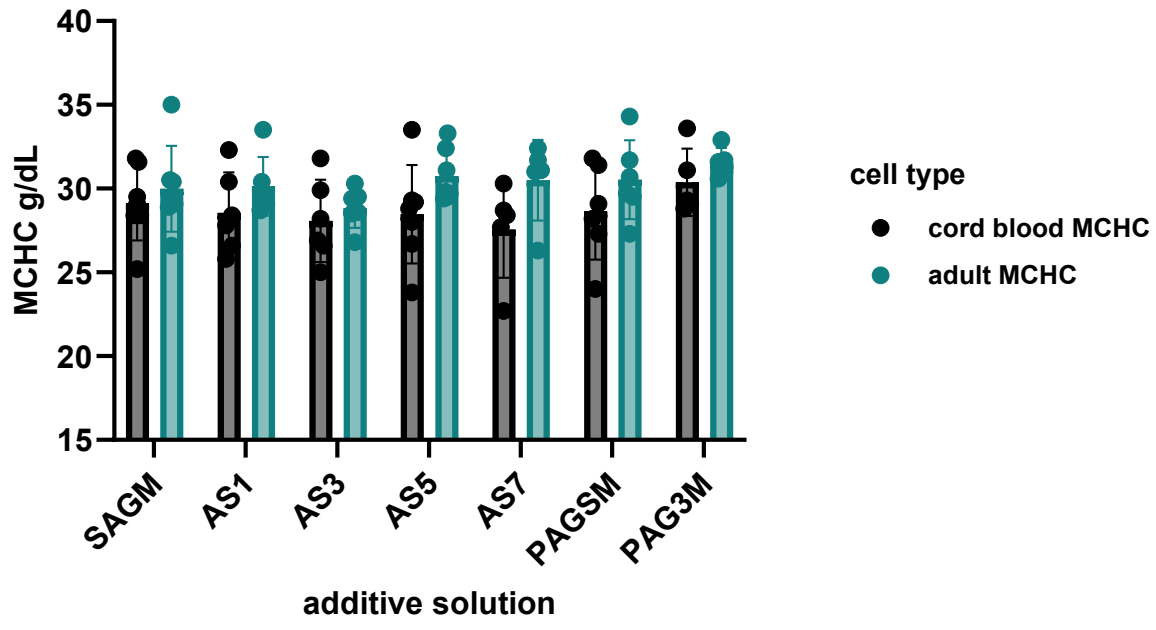

***Figure 8**: MCHC (g/dL) of adult and cord blood erythrocytes after 24 h refrigerated storage and washing. MCV was assessed across the different additive solutions. Data are shown as individual values with mean ± SD (n = 7).*

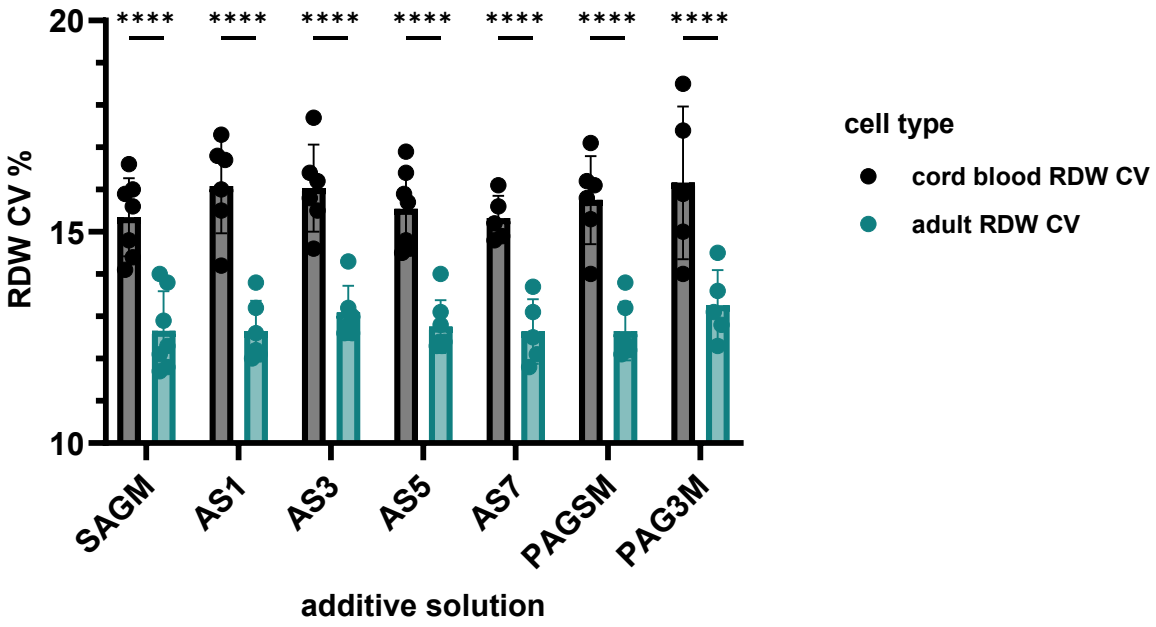

***Figure 9:** RDW CV (%) of adult and cord blood erythrocytes after 24 h refrigerated storage and washing. MCV was assessed across the different additive solutions. Data are shown as individual values with mean ± SD, (n = 7). Statistical significance is indicated as *p.adj < 0.05; **p.adj < 0.01; ***p.adj < 0.001; ****p.adj < 0.0001.*

3.3 Haemolysis over time

Haemolysis of red blood cells was assessed over four weeks in seven different additive solutions for both cord blood and adult samples. To account for differences in total haemoglobin content, values were normalised prior to analysis. Lower haemolysis was considered indicative of improved storage quality. Samples were washed after 24 h of storage to remove haemolysis caused by sample processing and preparation procedures. Therefore, the first haemolysis values shown in the time-course analysis represent week one.

At the initial time point (week zero, prior to washing), neonatal erythrocytes stored in AS7 displayed the highest haemolysis. However, two-way ANOVA with post hoc analysis using Šídák's multiple comparisons test) revealed no statistically significant differences in haemolysis between any of the additive solutions at this time point (all comparisons: adj. p-value > 0.05) (Figure 10). In adult erythrocytes, AS7 and PAGSM showed the highest haemolysis at baseline. However, no statistically significant differences were observed between any of the additive solutions (all comparisons: adj. p-value > 0.05).

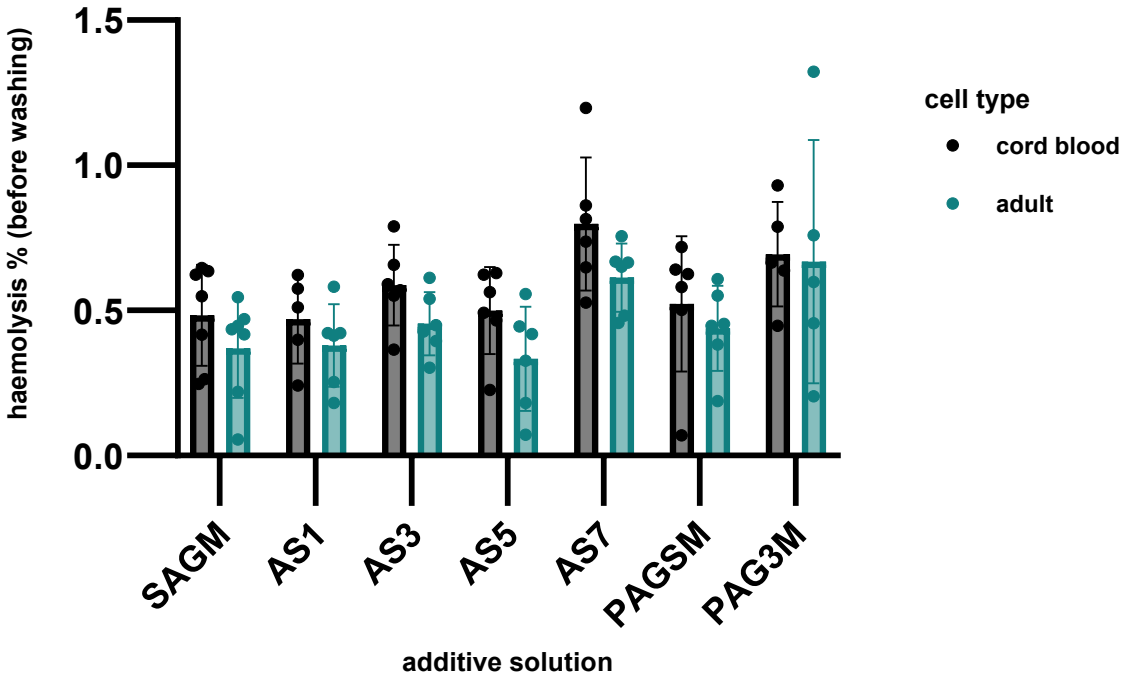

Figure 10: *Haemolysis (%) of cord blood and adult erythrocytes at week zero, before washing. Data are presented as mean ± SD. Statistical significance is indicated as *p.adj < 0.05; **p.adj < 0.01; ***p.adj < 0.001; ****p.adj < 0.0001.*

As expected, haemolysis increased over time irrespective of the additive solution and the sample origin. As shown in figure 11, SAGM exhibited the highest haemolysis in cord blood, with a mean value of 2.73% (n = 7), after four weeks of cold storage. This value was notably higher than that observed from cells stored in other solutions, indicating that SAGM, despite its widespread usage, does not constitute the optimal biological choice. In contrast, PAG3M showed the lowest haemolysis in CB, with a value of 1.27% (mean, n =6), closely followed by AS7 (mean = 1.38, n = 6) and PAGSM (mean = 1.39, n = 6) A similar trend was observed in adult samples, where SAGM also showed the highest haemolysis (mean = 1.91%, n = 6), while PAG3M demonstrated the lowest haemolysis (mean = 1.16%, n = 6). The haemolysis values observed in adult cells stored with AS1, AS3, AS5, AS7 and PAGSM were similar, with a mean deviation of ±0.2% and a total range of 0.4% between the highest and lowest values. In general, the haemolysis values were rather comparable across sample origin, with the exception of SAGM. REML-based mixed effects analysis revealed a significant effect of time on haemolysis (p-value < 0.0001). However, no significant overall difference between cord blood and adult erythrocytes was detected (p-value = 0.5159), nor was a significant interaction between sample origin and time (p-value = 0.0990) evident. Post hoc analysis using Tukey's multiple comparisons test identified a significant

difference between cord blood and adult erythrocytes in SAGM at week four (adj. p-value 0.036).

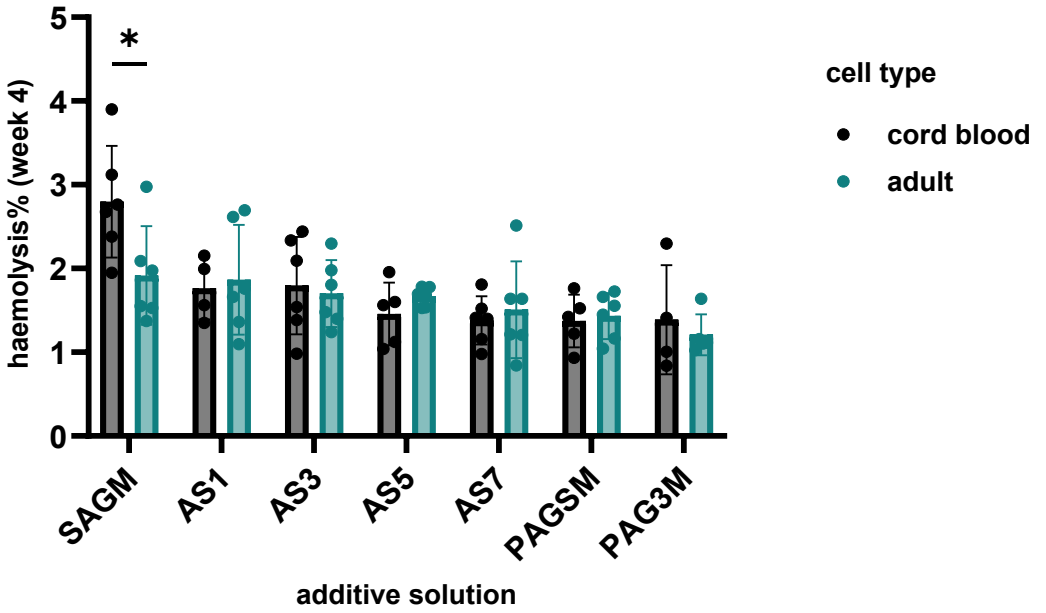

Figure 11: *Haemolysis levels of adult erythrocytes stored in different additive solutions after four weeks of storage. Data are presented as mean ± SD (n = 6).Statistical significance is indicated as *p.adj < 0.05; **p.adj < 0.01; ***p.adj < 0.001; ****p.adj < 0.0001.*

Over time, haemolysis increased in all additive solutions, with the most pronounced changes generally occurring between week three and week four in both cord blood (Figure 12) and adult samples (Figure 13). In SAGM-stored adult samples (Figure 13), exhibited the steepest increase was observed between week three and week four. For AS1, the haemolysis profile in cord blood (Figure 12) was relatively linear over time, while adult samples (Figure 13) showed a more pronounced increase between week two and week three. REML-based mixed effects analysis for cord blood, revealed a significant effect of additive solution on haemolysis in cord blood erythrocytes (p-value < 0.0001). Post hoc analysis (Tukey's multiple comparisons test) revealed no significant differences between any of the additive solutions at week one, two and three (adj. p-value> 0.05). At week four, however, SAGM-stored cord blood erythrocytes exhibited significantly higher haemolysis compared to AS7 (adj. p-value = 0.0200), PAGSM (adj. p-value = 0.00666), and PAG3M (p = 0.0174). In adult erythrocytes, REML-based mixed effects analysis similarly revealed a significant effect of additive solution on haemolysis (p-value = 0.0002). Post hoc analysis (Tukey's multiple

comparisons test) revealed no significant differences between any of the additive solutions at any time point (adj. p-value> 0.05).

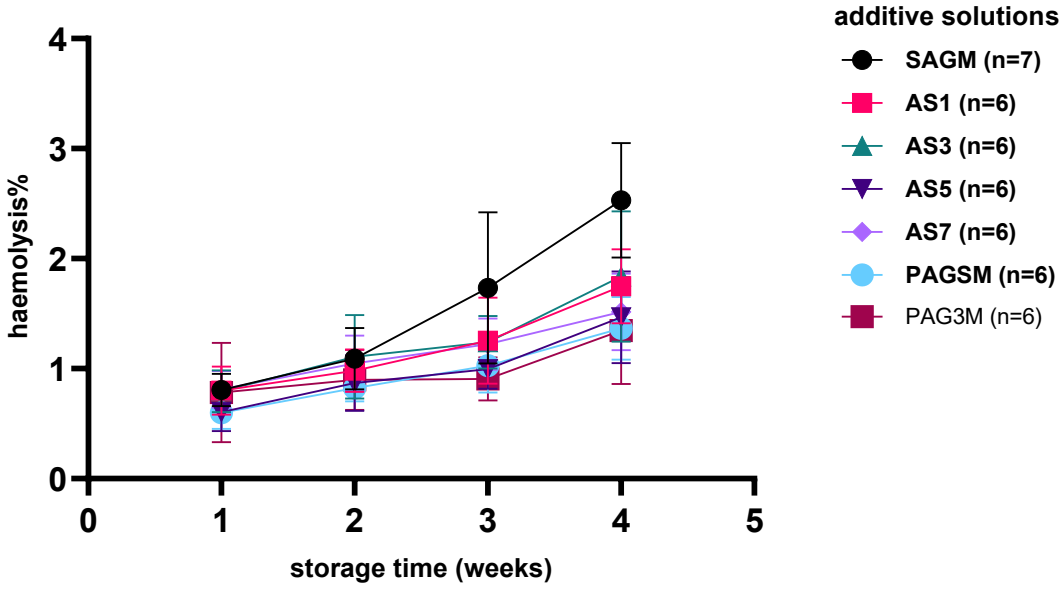

Figure 12: *Time-dependent haemolysis (%) of cord blood erythrocytes over four weeks of cold storage in seven different additive solutions. Data are shown as mean ± SD.*

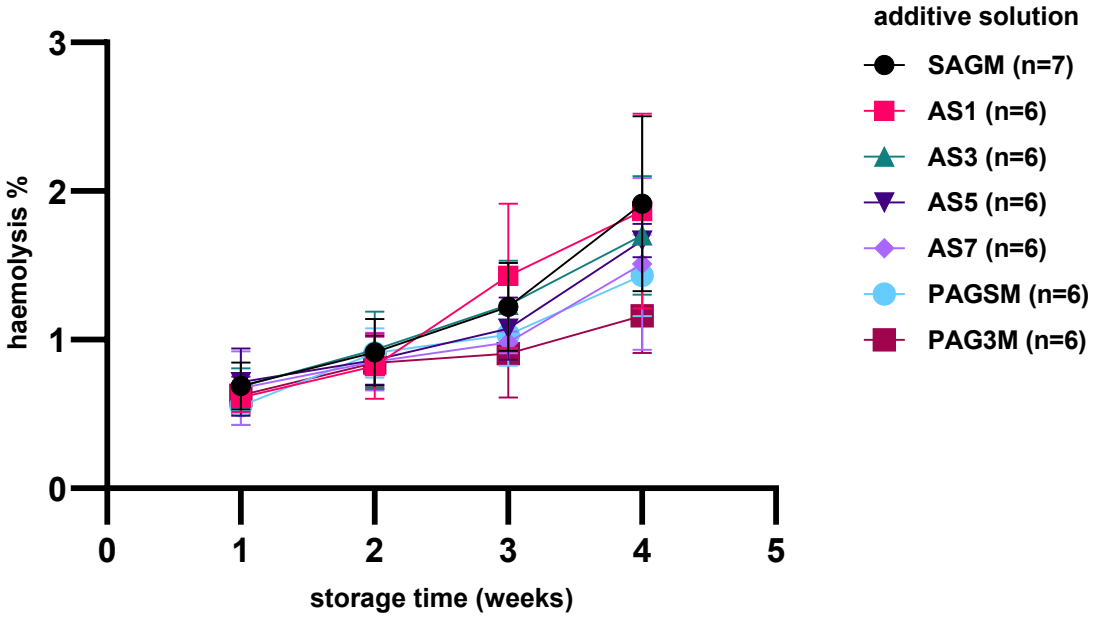

Figure 13: *Time-dependent haemolysis (%) of adult erythrocytes over four weeks of cold storage in seven different additive solutions. Data are shown as mean ± SD.*

When comparing the time course between cord blood and adult samples, it became apparent that AS3 and PAG3M displayed very similar haemolysis profiles in both groups. In contrast, AS1 and AS5 showed consistently higher haemolysis levels in cord blood, although the overall trends remained comparable. More pronounced differences between the two groups were observed in solutions AS7, where haemolysis of cord blood samples was consistently higher than in adult samples; however, between week three and week four, the increase in haemolysis was greater in adult cells than in cord blood.

To assess the effect of donor-specific variability on haemolysis, the area under the curve (AUC) of haemolysis over the storage period was calculated for each donor and additive solution. AUC values for respective solutions were ranked for each donor to visualise how the donor responded to the storage conditions. Rank 1 represents the lowest cumulative haemolysis within the respective solution, whereas higher ranks indicate higher cumulative haemolysis (Figure 14). In adult erythrocytes, the AUC ranking revealed pronounced inter-donor variability. Donor 7 showed consistently higher cumulative haemolysis across several additive solutions, whereas donor 8 displayed generally lower haemolysis ranks. This indicates that haemolysis during storage was strongly influenced by donor-specific factors. A similar donor-dependent pattern was observed in umbilical cord blood erythrocytes. The ranking showed substantial variability between cord blood donors, with donor 6 exhibiting higher cumulative haemolysis across several storage conditions and donor 2 showing lower overall ranks. In addition, the relative ranking of individual additive solutions differed between donors, suggesting that donor-specific characteristics influenced the response to the respective storage condition. Moreover, no clear pairing of experimental batches was visible in the data, indicating that donor biology not sample processing influenced the observed patterns.

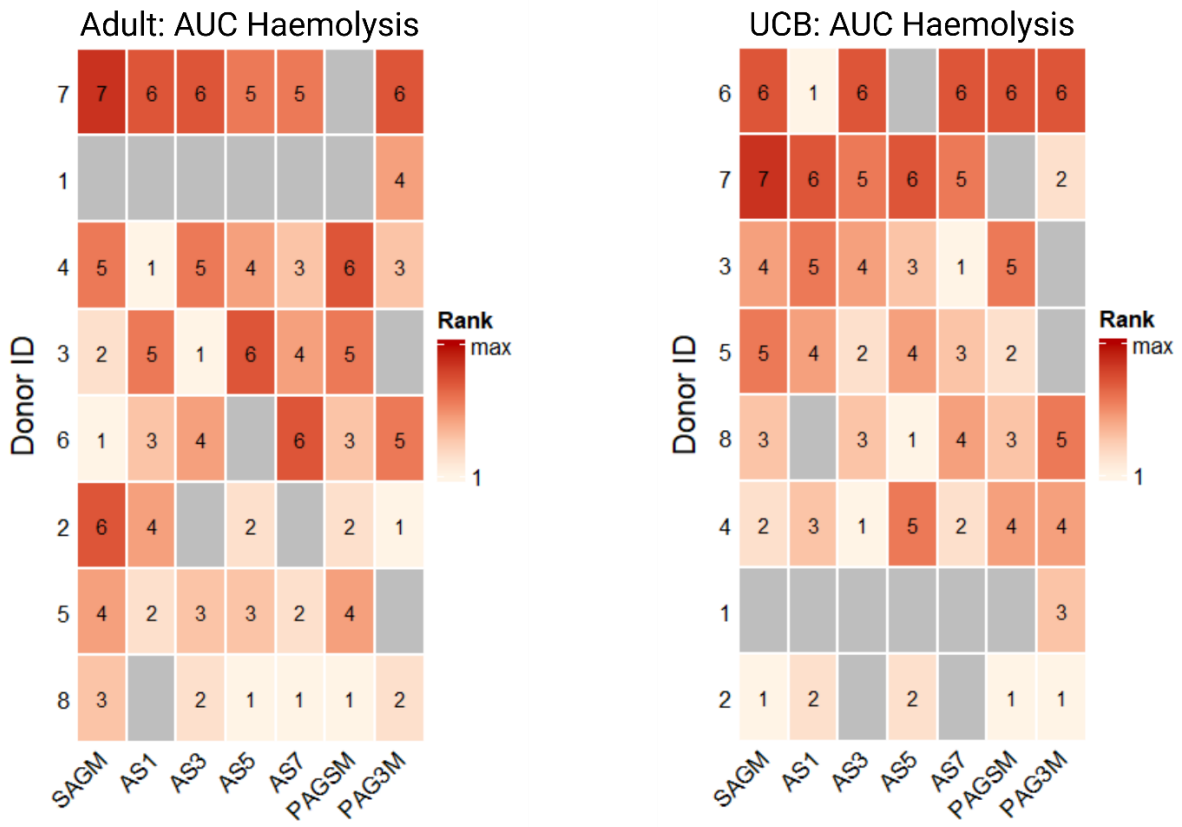

Figure 14: *Donor-dependent variability in haemolysis for adult (A) and umbilical cord blood (B) erythrocytes. Haemolysis over the storage period was summarised by calculating the area under the curve (AUC) for each donor and additive solution. Rank 1 indicates the lowest cumulative haemolysis within the respective solution, whereas rank 7 (SAGM) or 6 (all other solutions) indicate the highest cumulative haemolysis for a given solution. Grey squares represent missing donor–solution combinations. Donors (rows) are ordered by their average rank across the evaluated conditions.*

3.4 Morphology

Microscopic analysis was performed on blood smears at week four to evaluate the morphology of adult and neonatal erythrocytes following cold storage in the different additive solutions. Overall, both adult and cord blood samples showed differences in erythrocyte morphology depending on the additive solution used. In general, a higher proportion of discocytes was accompanied by lower levels of sphero- and echinocytes, indicating better preservation of erythrocyte morphology and adequate veracity of our methodology.

In adult RBCs, AS7 showed the highest proportion of morphologically normal erythrocytes (discocytes, mean = 76.4%, n = 6) and the lowest incidence of morphological abnormalities, including echinocytes (22.6%) and spherocytes (1.0%) (Figure 15). PAG3M demonstrated a comparable overall morphology profile but showed a slightly lower proportion of discocytes and higher proportions of echinocytes

and spherocytes. PAGSM-stored cells exhibited a substantially lower proportion of discocytes (44.2%) alongside higher proportions of echinocytes (42.7%) and spherocytes (13.1%), indicating a less favourable morphological profile compared to AS7 and PAG3M. In contrast, SAGM, AS1 and AS5 showed considerably lower discocyte levels and increased proportions of echinocytes and spherocytes. Strikingly, AS3 samples contained more echinocytes than discocytes and a considerably higher amount of spherocytes (26.4%) compared with the other additive solutions.

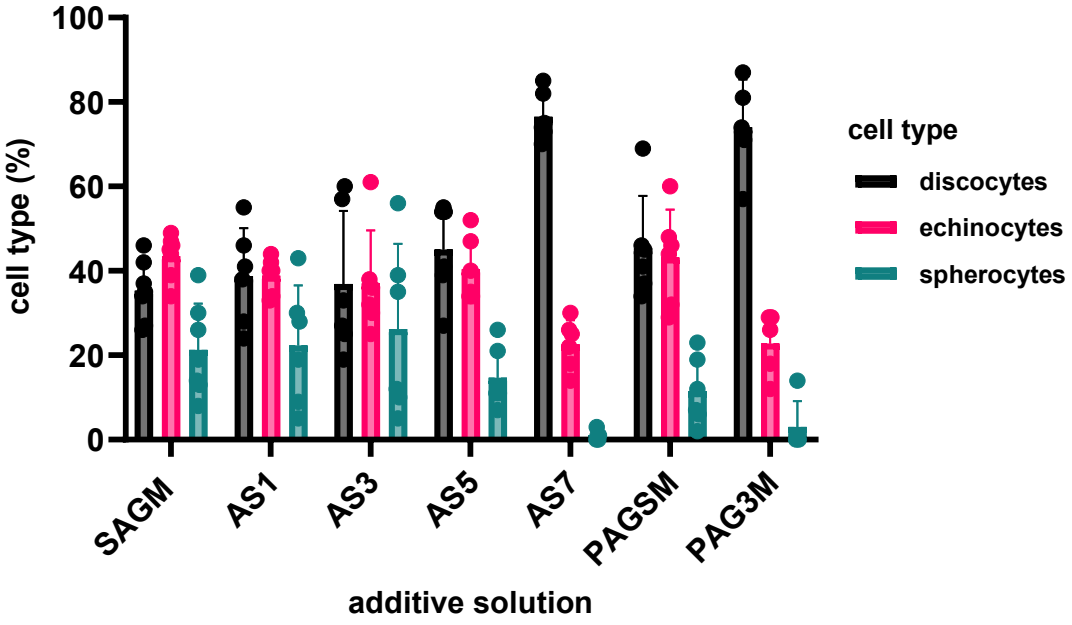

Figure 15: *Discocytes, echinocytes and spherocytes (%) in adult red blood cells after four weeks of cold-storage in seven different additive solutions. Data are shown as mean ± SD.*

A similar trend was observable in cord blood samples (Figure 16). AS7 and PAG3M resulted in the highest proportion of shape-preserved cells (discocytes, mean 73.9% and 63.8%, respectively) and the lowest amount of spherocytes (mean = 0.1% and 2.2%, respectively).

However, several differences compared to adult RBCs became apparent (Figure 17). REML-based mixed effects analysis with post hoc testing (Tukey's multiple comparisons test) revealed no statistically significant differences between cord blood and adult erythrocytes in any of the additive solutions (all $p > 0.05$). Nevertheless, several numerical differences were apparent. In particular, AS1 and SAGM exhibited noticeably higher proportions of discocytes and lower levels of

spherocytes compared to their adult counterparts. AS1 in cord blood showed a mean of 50.1% (n = 6) discocytes, whereas only 38.7% discocytes were observed in adult red blood cells. Likewise, neonatal cells stored in SAGM maintained a higher proportion of discocytes (mean = 45.5%, n = 7) and lower levels of spherocytes (mean = 21.2%, n = 7) compared to adult ones.

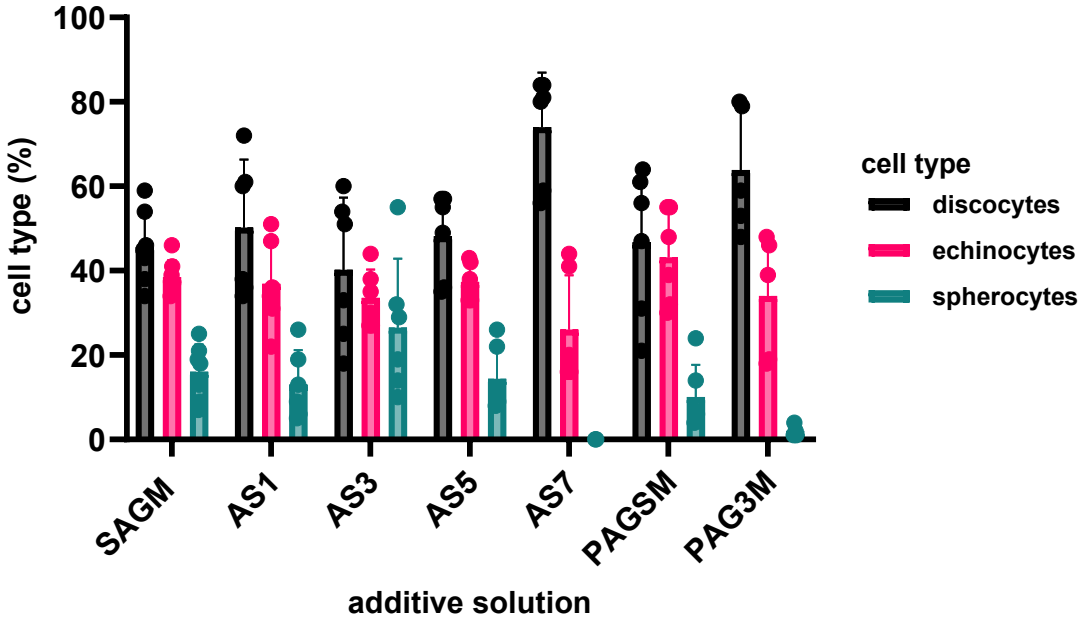

***Figure 16**: Discocytes, echinocytes and spherocytes (%) in neonatal red blood cells after four weeks of cold-storage in seven different additive solutions. Data are shown as mean ± SD.*

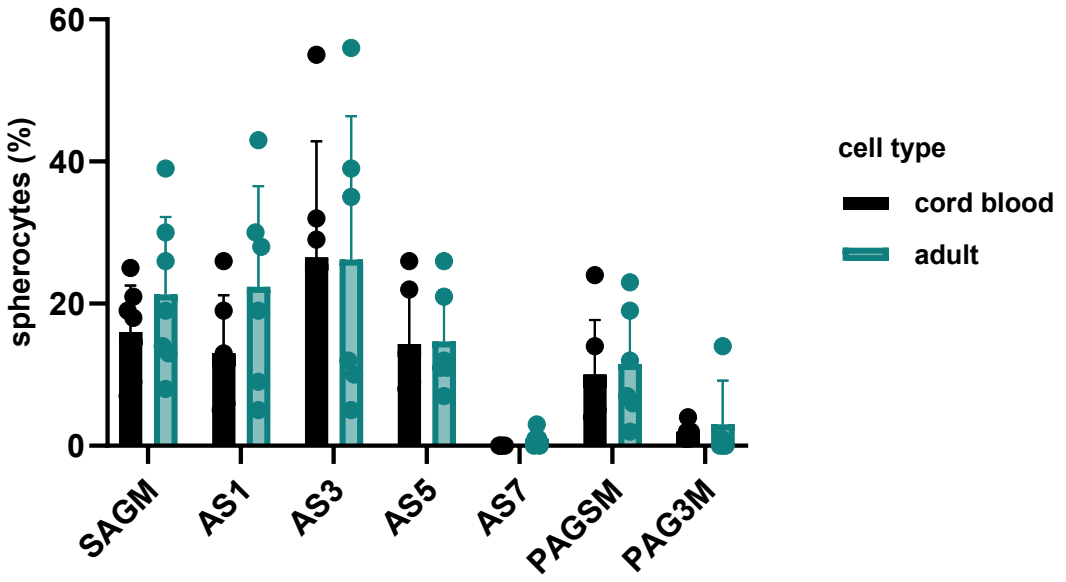

***Figure 17**: Spherocytes(%) in cord blood and adult red blood cells after four week of cold-storage in seven different additive solutions. Data shown as mean ± SD.*

3.5 Biochemical

3.5.1 Lactate

To determine intracellular lactate concentrations, stored red blood cells were precipitated, and the resulting supernatant used for analysis. Lactate was measured on a spectrophotometer at 340 nm. Measurements were performed weekly over a period of four weeks across all additive solutions for both adult and neonatal erythrocytes.

Among adult samples, the highest intracellular lactate concentrations were observed in cells stored in AS7 and PAG3M, followed by PAGSM at an intermediate level. AS5, AS1 and SAGM-stored cells formed a close cluster and displayed lower values than cells stored in alkaline solutions, while cells stored in AS3 exhibited the overall lowest lactate production (Figure 18). REML-based mixed effects analysis revealed significant effects of both additive solution (p-value < 0.0001) and time (p-value < 0.0001) on lactate concentration, as well as a significant interaction between the two factors (p-value < 0.0001), indicating that differences between additive solutions became more pronounced over the storage period. Post hoc analysis (Tukey's multiple comparisons test) confirmed that AS3 showed significantly lower lactate concentrations compared to almost all other solutions across all time points (adj. p-value < 0.05), while AS7 and

PAG3M exhibited significantly higher values than SAGM, AS1, AS3, and AS5 at most time points (adj. p-value < 0.05). SAGM, AS1, and AS5 did not differ significantly from one another at any time point (adj. p-value > 0.05). These findings were mirrored in cord blood erythrocytes, where the same significant effects of additive solution, time, and their interaction were observed (adj. p-value < 0.0001).

When directly comparing lactate production kinetics between adult and neonatal red blood cells across the four-week observation period, a largely consistent pattern emerged. SAGM, AS7, and PAG3M showed nearly identical values between their respective adult and neonatal counterparts throughout the entire period. AS3 followed a similar trajectory in both groups, though by week four cord blood RBCs slightly exceeded the lactate production of adult cells. Similarly, lactate production in PAGSM-stored cord blood erythrocytes also diverged from adult values toward the end of the observation period. For AS1, lactate measured in cord blood was marginally higher than in adult cells by the final week.

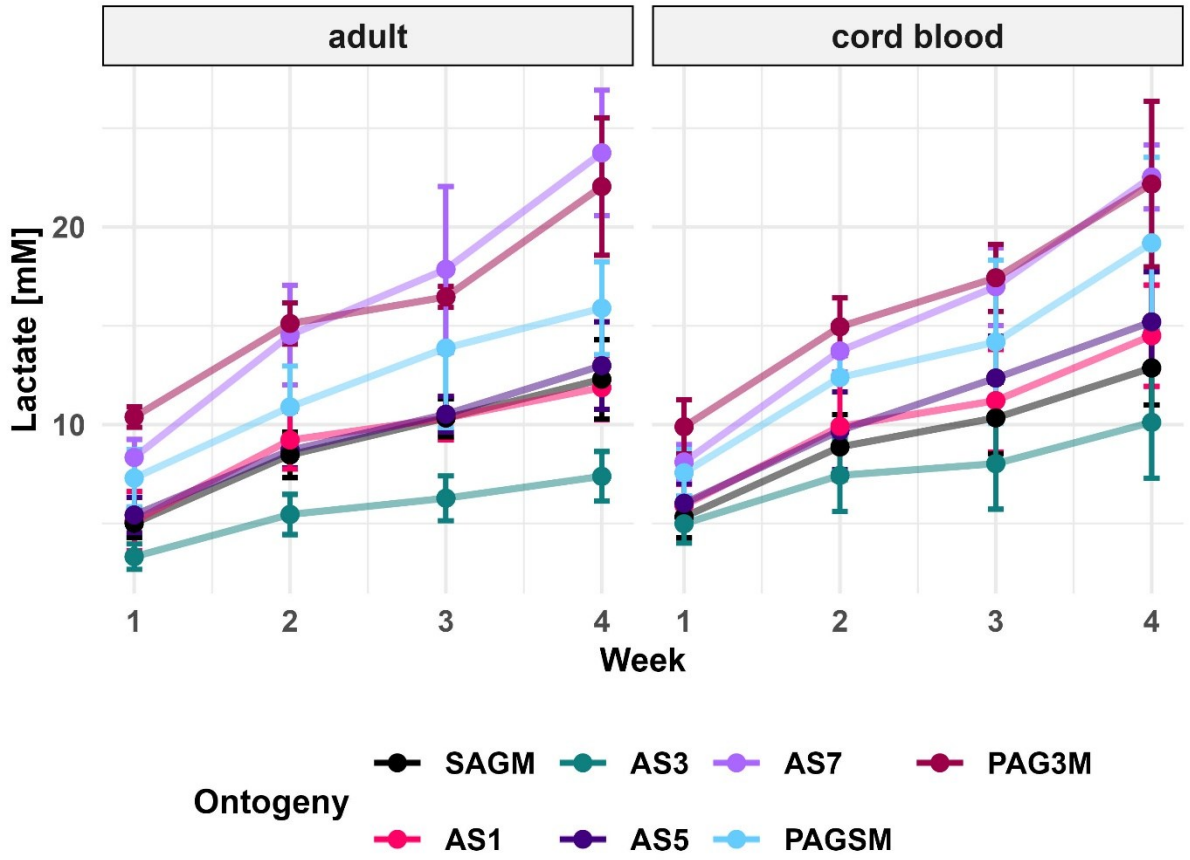

Figure 18: *Time course of intracellular lactate [mM] concentration over a 4-week period in cord blood and adult red blood cells. Data are shown as mean with ± SD.*

Interestingly, when examining the lactate production of each sample over the course of four weeks of cold storage between cord blood and adult erythrocytes, distinct patterns emerged (Figure 19). In cord blood, the largest increases were observed in PAGSM, PAG3M, and AS3, followed by SAGM, with AS1, AS5, and AS7 showing comparable but smaller changes. Adult red blood cells, showed the greatest increase in AS7, followed by AS5 and SAGM, then PAGSM, then PAG3M, and finally subjects AS1 and AS3 with the smallest changes. REML-based mixed effects analysis with post hoc testing (Tukey's multiple comparisons test) confirmed no significant difference between cord blood and adult erythrocytes in any of the additive solutions (p-value> 0.05).

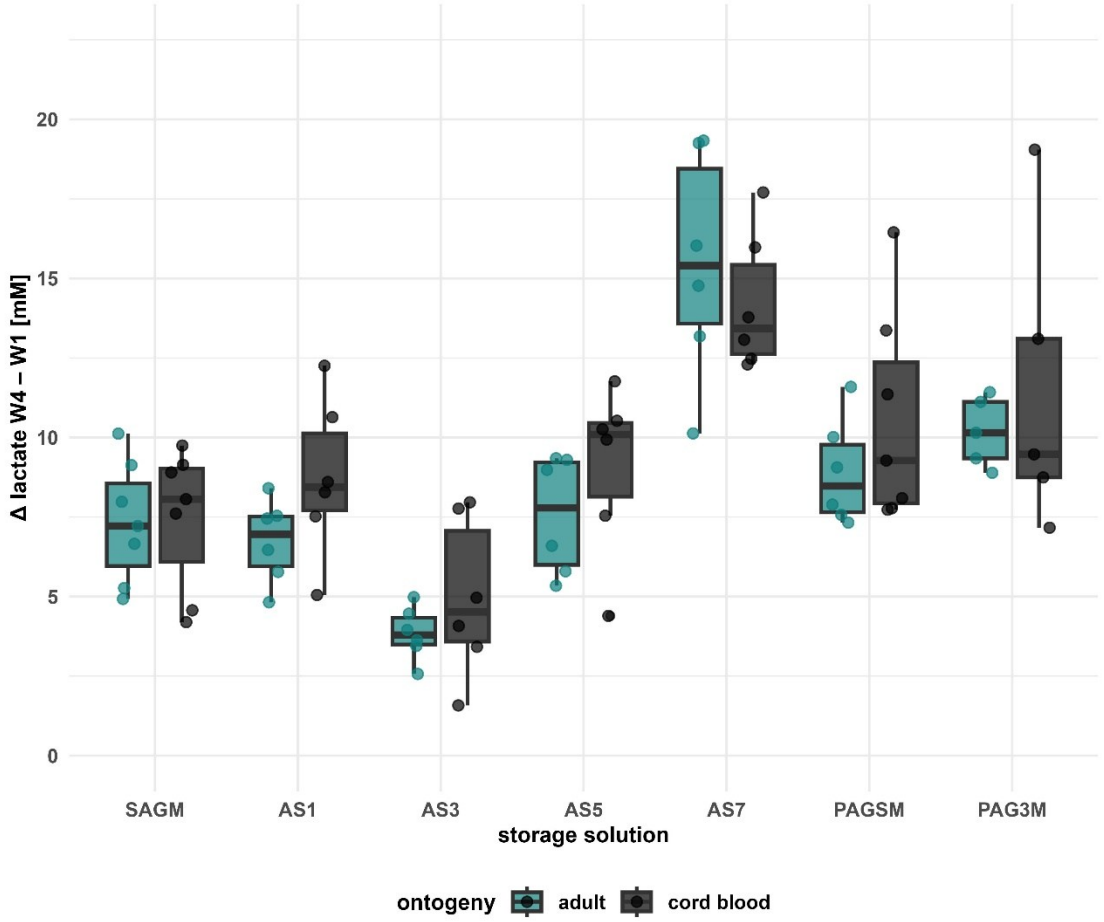

***Figure 19**: Comparison of lactate production [mM] across four weeks of cold storage between cord blood and adult red blood cells stored in the various additive solutions. Data are presented as boxplots for each additive solution and group, showcasing the median, interquartile range, and overall distribution of differences in intracellular lactate concentrations..*

3.5.2 Glucose

To determine intracellular glucose concentrations, stored red blood cells were precipitated, and the resulting supernatant used for analysis. Glucose was measured on a plate-reader at 340 nm using. Measurements were performed weekly over a period of four weeks across all additive solutions for both adult and neonatal erythrocytes.

Adult red blood cells stored in AS1, exhibited the highest initial glucose concentration and also showed the greatest overall decline over the 4-week period. They consistently maintained the highest glucose concentrations throughout, ranging between 63.24 mM and 53.43 mM. AS7-stored adult red blood cells followed, with an initial value of 44.7 mM and a final value of 37.1 after four weeks. Interestingly in AS7, the most

pronounced drop was observed between week two and three, whereas the change between week three and four was negligible. AS3 ranked next, showing little change between week one and two, followed by a more notable decline from week two onwards. SAGM, AS5, PAGSM and PAG3M, behaved very similarly to one another, displaying only a modest overall decline (Figure 20). REML-based mixed effects analysis confirmed a significant effect of additive solution on lactate concentration in adult erythrocytes (p-value < 0.0001). Post hoc analysis (Tukey's multiple comparisons test) identified significant differences between almost all additive solutions at week one, with the exception of SAGM vs. AS5, SAGM vs. PAGSM, and AS5 vs. PAGSM (adj. p-value> 0.05).

Neonatal erythrocytes showed a very similar pattern to adult cells with the exception of AS7-stored red blood cells. CB cells stored in AS7, displayed a more linear decline compared to their adult counterparts. Cord blood cells stored in AS3 behaved identically to adult cells. Notably, across all additive solutions, initial glucose concentrations in cord blood tended to be slightly higher than in adult erythrocytes. REML-based mixed effects analysis with post hoc testing (Tukey's multiple comparisons test) revealed that the pattern of significant and non-significant differences between additive solutions in cord blood largely mirrored that observed in adult erythrocytes, with only minor deviations at individual time points (adj. p-value> 0.05)

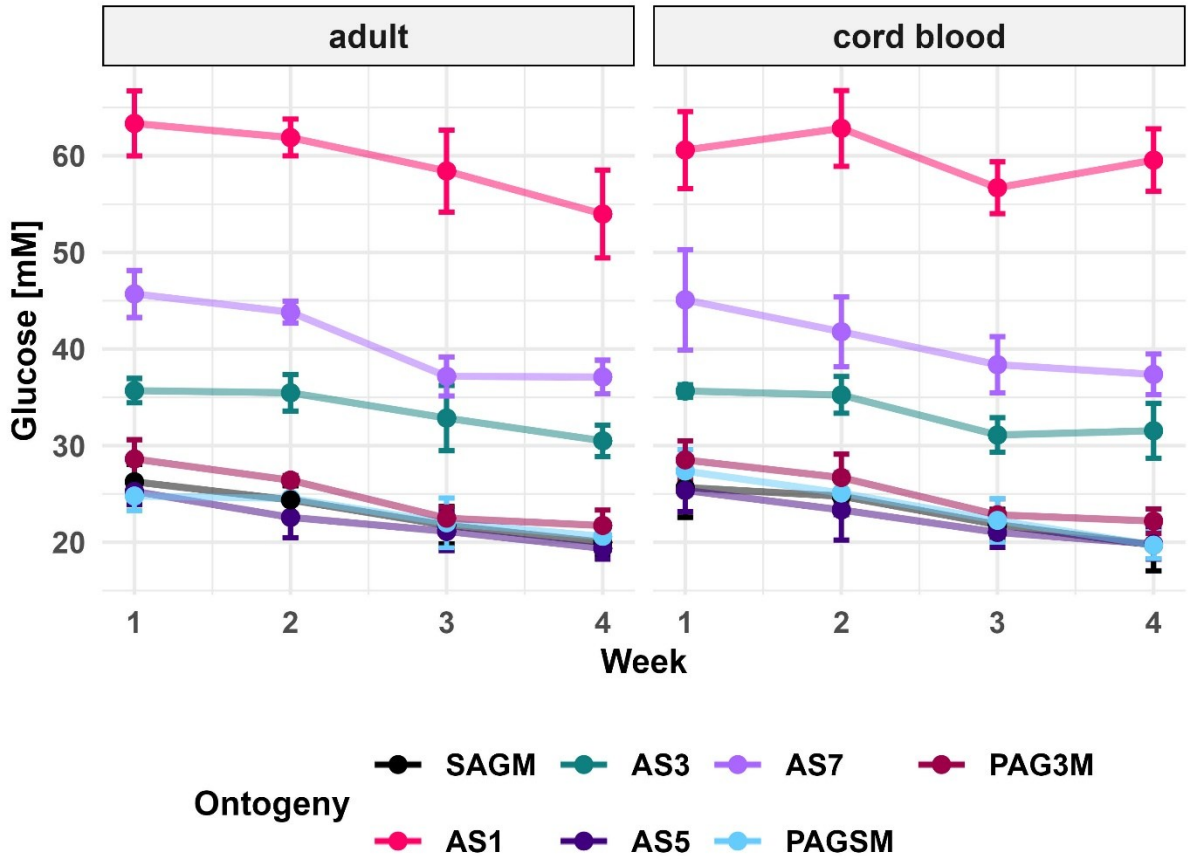

***Figure 20**: Time course of glucoses [mM] concentration over a 4-week period in cord blood and adult red blood cells. Data are shown as mean with ± SD*

When comparing the magnitude of glucose decline between week one and week four, notable differences were observed both between additive solutions and between cord blood and adult cells (Figure 21). Overall, glucose decreased in all additive solutions, as indicated by the predominantly negative delta values, reflecting ongoing glycolytic activity throughout the storage period. SAGM, AS3, AS5, and PAGSM showed a consistently greater glucose reduction in cord blood compared to adult erythrocytes, suggesting higher metabolic activity in neonatal red blood cells under these conditions. In contrast, AS7 and PAG3M exhibited a larger glucose decline in adult erythrocytes, indicating solution-dependent differences in metabolic behaviour between the two ontogenetic groups. AS1 stands out as a notable exception, cord blood showed a median delta close to zero with high variability, suggesting inconsistent or limited glucose consumption, while adult erythrocytes displayed a substantially greater decline. However, REML-based mixed effects analysis revealed no statistically

significant differences between cord blood and adult erythrocytes in any of the additive solutions (adj. p-value> 0.05).

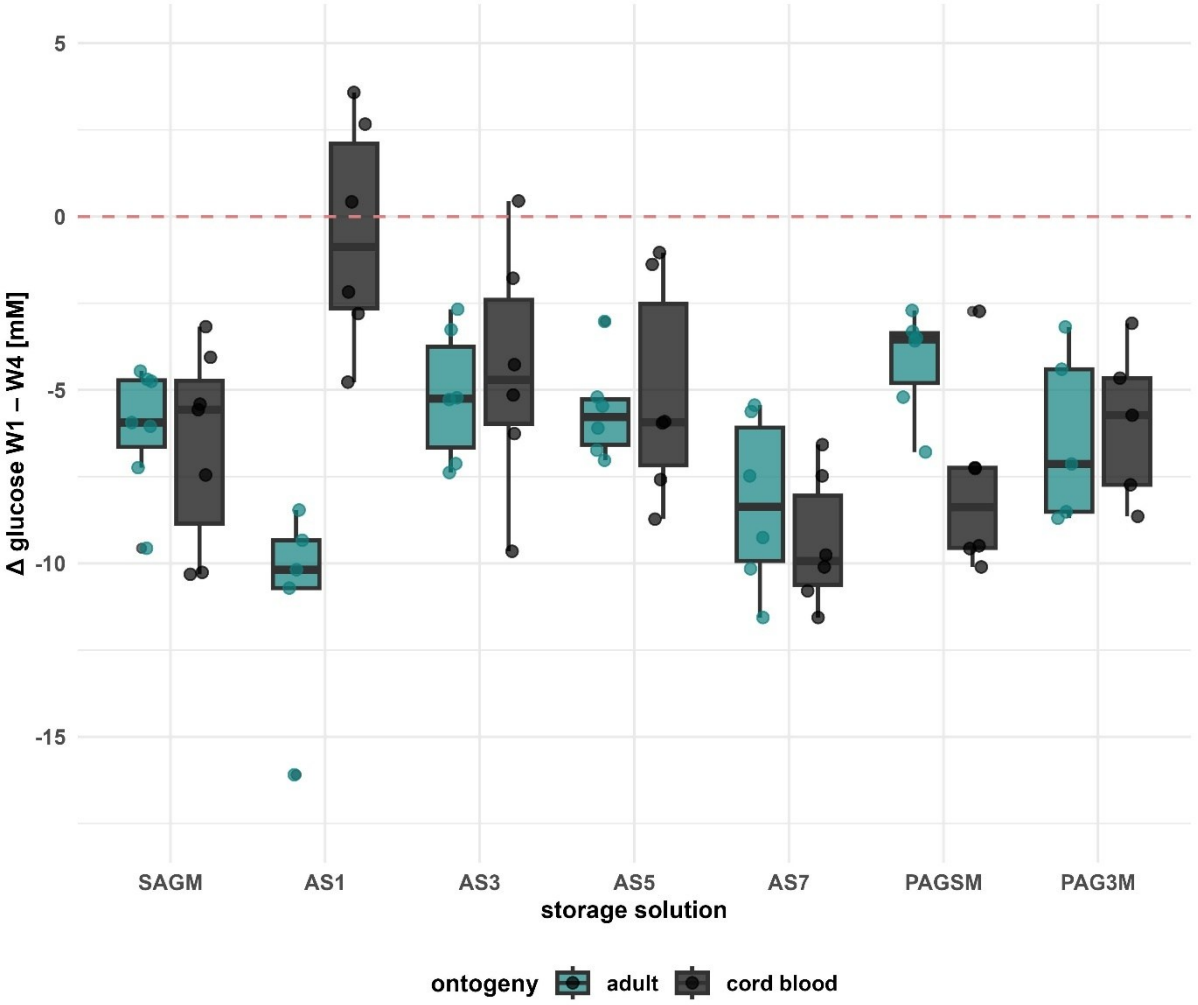

Figure 21: *Comparison of intracellular glucose decline [mM] across four weeks of cold storage between cord blood and adult red blood cells stored in various additive solutions. Data are presented as boxplots for each solution and group, showcasing the median, interquartile range, and overall distribution of differences in intracellular glucose concentrations.*

3.6 Correlation Analysis

Spearman rank correlation analysis was performed to examine the relationships between RBC storage characteristics, haemolysis, spherocyte formation, intracellular lactate and glucose concentrations, and the biochemical composition of the additive solutions, in both cord blood and adult erythrocytes. Correlation coefficients were classified as strong ($|r| \geq 0.7$), moderate ($0.5 \leq |r| < 0.7$), or weak ($0.3 \leq |r| < 0.5$). The correlation is shown as a heatmap in figure 23.1

A notable difference between the two erythrocyte sources was observed in the relationship between haemolysis and spherocyte formation. In adult erythrocytes, these two parameters were significantly and positively correlated ($r = 0.506$, $p = 0.001$), indicating that solutions associated with greater haemolysis also tended to promote spherocyte formation. In cord blood erythrocytes, however, no such correlation was found ($p = 0.272$), suggesting that in CB membrane rupture and morphological remodelling may represent more independent processes.

In contrast, the inverse relationship between spherocyte formation and lactate was a consistent finding across both sources. In adult erythrocytes, this correlation was moderate and negative ($r = -0.632$, $p < 0.001$), and a comparable pattern was observed in cord blood ($r = -0.437$, $p = 0.007$). Similarly, haemolysis was negatively associated with lactate in both groups, suggesting that solutions supporting higher glycolytic activity were associated with better preservation of RBC membrane integrity regardless of erythrocyte source. Glucose showed no significant correlations with any quality marker in adult erythrocytes, whereas in cord blood a moderate positive association with spherocyte formation was observed ($r = 0.596$, $p < 0.001$).

Several constituents showed consistent associations with storage characteristics across both erythrocyte sources. Mannitol was among the most consistently protective constituents, showing negative correlations with both haemolysis and spherocyte formation in adult erythrocytes ($r = -0.397$ and $r = -0.616$, respectively; both $p \leq 0.010$) and in cord blood ($r = -0.562$ and $r = -0.473$; both $p \leq 0.002$). Similarly, Na_2HPO_4 was negatively associated with haemolysis and spherocyte formation in both groups, and sodium chloride showed a consistent weak positive correlation with haemolysis in both adult and cord blood erythrocytes.

Lactate accumulation followed a broadly consistent pattern across both sources. In both adult and cord blood erythrocytes, lactate showed strong positive correlations with mannitol, the strongest single association observed in both datasets (adult: $r = 0.772$, CB: $r = 0.669$; both $p < 0.001$), as well as with Na_2HPO_4 and $NaHCO_3$. Negative correlations with citrate and sodium citrate were equally consistent across both groups, with comparable effect sizes.

An elevated additive solution pH was negatively associated with haemolysis and spherocyte formation in both erythrocyte sources, and positively associated with lactate, suggesting that a more alkaline environment consistently supports glycolytic activity while attenuating storage-induced membrane deterioration. This pattern was observed across both adult and cord blood erythrocytes, pointing to pH as a particularly relevant parameter in the design of additive solutions.

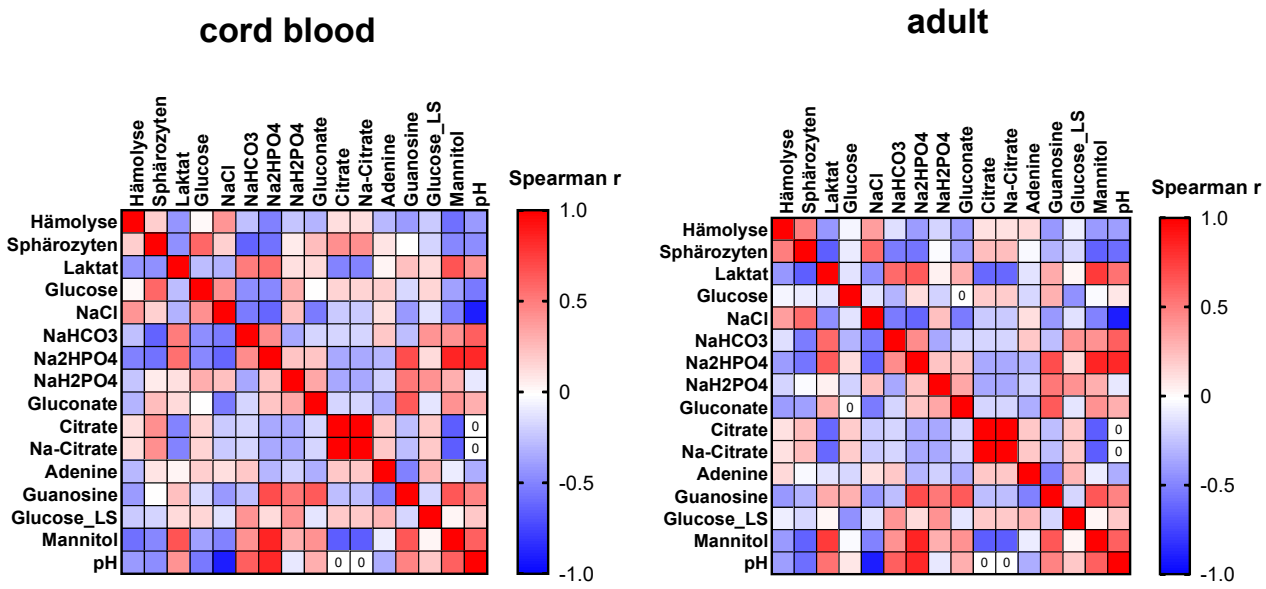

Figure 22: *Heatmaps display Spearman correlation coefficients (r) between RBC quality markers (haemolysis, spherocyte formation, lactate, glucose) and additive solution constituents for cord blood (left) and adult erythrocytes (right). Colour intensity reflects the strength of the correlation, with red indicating positive and blue indicating negative associations. Cells marked with "0" indicate a correlation coefficient of zero or no calculable correlation due to invariant values across samples.*

4 Discussion

The present study aimed to investigate the storage behaviour of neonatal and adult erythrocytes across seven different additive solutions over a four-week period. The findings broadly confirm that the choice of additive solution significantly influences storage quality, and that neonatal and adult erythrocytes behave largely similarly during storage, with differences confined to specific aspects, most notably at the morphological level. These observations can likely be explained by intrinsic biological differences between the two cell populations

One of the most striking findings was that SAGM consistently showed the highest haemolysis in both cord blood and adult samples. This suggests that factors in other additive solutions may beneficially alter storage trajectories. There could be several reasons why haemolysis is higher in SAGM than in the other additive solutions. Firstly, SAGM contains no phosphate groups (63). All additive solutions, used in the present work, which contained phosphate, exhibited lower haemolysis. Phosphate is essential for ATP synthesis, and ATP itself plays a central role in erythrocyte metabolism and membrane stability (80). Absent phosphate availability could contribute to the increased haemolysis observed in this additive solution (66). In addition, the relatively low pH value of SAGM (pH = 5.7) may further impair erythrocyte stability. Acidic storage conditions can promote oxidative stress and reactive oxygen species formation, thereby contributing to membrane damage and haemolysis (66). Furthermore, low pH values may impair enzyme activity and consequently reduce ATP availability. Yoshida et al. demonstrated that red blood cells stored in SAGM undergo a rapid decline in 2,3-DPG within the first days of storage, accompanied by a progressive decrease in ATP and pH. As a consequence, ATP-dependent membrane transport systems such as the Na+/K+ pump and Ca2+ pump can no longer function properly (66). In addition to these pH-associated effects, metabolic alterations indicative of oxidative stress have also been observed in SAGM-stored erythrocytes. d'Alessandro et al. demonstrated that red blood cells stored in SAGM exhibited relatively high GSSG levels already early during storage, suggesting an increased oxidative burden (85). These metabolic and oxidative alterations are accompanied by more global structural membrane changes during storage. In SAGM, microparticle formation, cytoskeletal damage and membrane inversion contribute to progressive membrane remodelling and ultimately promote the formation of spherocytes (66).

Although overall trends were similar, neonatal erythrocytes stored in SAGM showed a higher haemolysis than adult ones. When comparing the components of SAGM with other additive solutions, it is notable that AS1 and AS5 are similar in composition, yet concentrations of individual components differ. However, AS1 as well as AS5 stored cells show considerably less haemolysis. Thus, it is interesting to note that SAGM includes the lowest amounts of mannitol and adenine. Given observable differences between adult and CB-cells, this may suggest that the two components exert specific beneficial effects on neonatal red blood cells. This theory is supported by the fact that

additive solutions that lead to the lowest haemolysis in cord blood cells continuously maintain these two ingredients at a higher concentration.

In contrast, alkaline additive solutions such as AS7, PAGSM and PAG3M showed the lowest haemolysis in both adult and neonatal red blood cells, as well as low proportions of spherocytes, suggesting that red blood cells benefit from more alkaline storage conditions. One key reason for this is the effect of pH on glycolytic activity. Previous studies demonstrated that alkaline additive solutions improve glycolytic activity and redox metabolism during storage by maintaining phosphofructokinase activity at higher intracellular pH values, thereby supporting ATP preservation and metabolic stability (66). In contrast, an acidic pH environment promotes ROS formation via the Fenton reaction and inhibits enzyme activity (66,95), which can lead to cell damage. As glycolysis during storage leads to progressive lactate accumulation and pH decline (96), the initially higher pH in these solutions delays the onset of these detrimental effects. However, the relatively higher lactate levels observed at week four may challenge this hypothesis, and pH measurement at the end of storage would be necessary to draw firm conclusions.

Beyond pH, the specific composition of these solutions likely contributes to their superior preservation capacity. All three solutions contain phosphate, which serves as a substrate for ATP and 2,3-DPG generation, thereby supporting metabolic stability and membrane integrity (85). Additionally, PAGSM and PAG3M contain adenine, which has been recognised since 1965 as a compound that increases intracellular ATP levels (88). It supports ATP regeneration via the purine salvage pathway by expanding the intracellular adenylate pool. However, a considerable proportion of adenine is eventually metabolised through purine degradation pathways, leading to the accumulation of hypoxanthine, a marker of oxidative stress and storage-associated damage (78). Guanosine, present in PAGSM and PAG3M, exerts its effects through a complementary mechanism: following cellular uptake, it is metabolised by purine nucleoside phosphorylase into guanine and ribose-1-phosphate, the latter entering the pentose phosphate pathway. Through the generation of glycolytic intermediates such as fructose-6-phosphate, this supports glycolysis and 2,3-DPG synthesis by increasing substrate availability for phosphofructokinase and thereby enhancing glycolytic flux. In addition, recycling of pentose phosphate pathway intermediates through the oxidative

branch of the pathway supports NADPH generation (42), thereby promoting glutathione recycling and antioxidative capacity (78,85). Notably, while adenine supplementation and 2,3-DPG preservation are typically inversely correlated, guanosine-containing solutions such as PAGSM and PAG3M appear to maintain both simultaneously, likely by compensating for adenine-induced ribose phosphate consumption (78,85). In PAG3M specifically, gluconate additionally promotes intracellular alkalinisation via the chloride shift, further activating glycolytic enzymes (85).

Overall, adult and neonatal erythrocytes showed largely comparable haemolysis patterns during storage, although isolated differences between the groups were observed. In contrast, more pronounced group-specific differences became evident at the morphological level. These observations can likely be explained by intrinsic biological differences between the two cell populations. Although reticulocytes represent only a minor fraction of cord blood cells (~6.4 ±1.5 %) (97), their ongoing ex vivo maturation may nonetheless contribute to these observed differences. Continued degradation of intracellular organelles requires ATP consumption and may promote ROS formation, potentially contributing to altered glucose consumption, increased vesiculation, and accelerated haemolysis during storage (97,98). In addition, the development of storage-related alterations is strongly driven by oxidative stress, which arises primarily from haemoglobin autoxidation and the progressive accumulation of reactive oxygen species during storage. These processes lead to lipid peroxidation and oxidative damage of membrane proteins, thereby contributing to progressive membrane destabilisation and morphological changes (66). These oxidative processes particularly affect membrane–cytoskeleton interactions. Oxidative modifications of structural proteins such as spectrin, actin, ankyrin, and band 3 have been shown to impair membrane stability and deformability, promoting morphological transitions from discocytes towards echinocytes and spherocytes (99). In parallel, erythrocytes rely on glutathione- and NAD(P)H-dependent antioxidant systems to counteract oxidative stress. However, during prolonged storage these protective mechanisms become progressively exhausted, which further enhances membrane instability and contributes to vesiculation as a stress response (66). Neonatal erythrocytes further showed higher activities of glycolytic and antioxidative enzymes, including G6PDH, which may contribute to improved ATP preservation, enhanced NADPH generation, and reduced

oxidative stress during storage (100). However, more plausible explanations for the observed differences also include structural and functional differences associated with foetal haemoglobin (HbF). The substitution of β- by γ-chains alters oxygen affinity and redox behaviour, and HbF has been suggested to interact less strongly with Band 3, potentially affecting metabolic regulation (100). These combined metabolic and structural differences may contribute to altered glucose consumption profiles and increased susceptibility to morphological changes in neonatal erythrocytes during storage. In addition, potential differences in cytoskeletal organisation and mechanical properties may further amplify these effects, as neonatal erythrocytes are generally larger and may deform differently under stress conditions (27).

D'Amici et al. reported less extensive membrane protein fragmentation in adult AS3 stored erythrocytes compared to SAGM, indicating a potentially lower degree of storage-associated membrane damage (63). As membrane fragmentation and vesiculation are linked to membrane surface loss, this could suggest a lower tendency for spherocyte formation. In contrast, the present study observed the highest proportion of spherocytes in AS3-stored erythrocytes, particularly in neonatal cells. However, haemolysis levels in AS3 remained within the intermediate range and were even slightly lower in CB than in adult ones. This phenomenon might indicate a more efficient metabolic or redox-related maintenance during storage compared to SAGM. Indeed, this has also previously been suggested by d'Alessandro et al., who described that citrate metabolism in erythrocytes may improve redox-associated pathways in AS3-stored erythrocytes (79). Although mature erythrocytes lack mitochondria and therefore do not possess a Krebs cycle, citrate can be metabolised through cytosolic TCA enzymes. This supports NADH and NADPH regeneration and promotes carbon flux through the pentose phosphate pathway. Since NADPH is required for glutathione recycling, this may improve redox homeostasis and thereby reduce oxidative damage to membrane and cytoskeletal proteins (79). Therefore, the present findings may suggest that morphological alterations and haemolysis do not necessarily develop in parallel during RBC storage.

Due to the low sample size, sex-related differences were not analysed in the present study. Previous studies demonstrated sex-related differences in RBC storage behaviour, particularly regarding redox homeostasis, haemolysis parameters and

membrane stability. Female donor-derived erythrocytes were shown to exhibit lower intracellular ROS accumulation and improved resistance against oxidative stress during storage, potentially associated with hormonal influences and differences in antioxidant enzyme activity (101). At the same time, differences in osmotic and mechanical fragility have also been described between male and female RBCs (101). This aspect may also be relevant for CB samples, as sex-related hormonal differences already emerge during the neonatal period and could potentially influence oxidative stress response and erythrocyte metabolism (102). Therefore, sex-related biological variability may have contributed to the observed differences in haemolysis and morphological alterations during storage and should be considered in future investigations. In addition, CB-derived erythrocytes may be affected by further donor-dependent factors that were not considered in the present study. Previous studies demonstrated that the lipid profile of neonatal erythrocytes is strongly influenced by maternal diet, genetic factors and placental transfer (27). Since membrane lipid composition contributes substantially to erythrocyte deformability and membrane stability (27), these factors may also affect susceptibility to storage-related alterations and haemolysis.

To the author's knowledge, no comparable studies have investigated seven different additive solutions across neonatal and adult erythrocytes. The present work may therefore serve as a foundation for ongoing research in the field of additive solution screening and for the translation of cord blood-derived red cell ATMPs into the clinical setting. Future studies should focus on bridging the gap between storage-related biochemical and morphological changes and clinically relevant outcomes. A first step would be the inclusion of larger and more diverse donor cohorts to account for biological variability, including sex-specific differences as well as donor-related factors such as age, health status, and for cord blood, gestational age and delivery characteristics. This would allow a more robust assessment of whether the observed differences between additive solutions and between cord blood and adult erythrocytes are consistent across broader populations. In addition, future studies should evaluate erythrocyte storage under routine blood bank conditions, including clinically relevant processing steps such as component preparation, irradiation, aliquoting, and varying storage durations. This would improve the external validity of the findings and better reflect real-world transfusion practice. As the present study focuses on surrogate

markers of storage lesions, functional endpoints should be incorporated in future work. These may include erythrocyte deformability, ATP and 2,3-DPG levels, endothelial interaction assays, oxygen delivery capacity, and other measures of cellular functionality that more directly reflect transfusion efficacy. Importantly, translational in vivo studies are required to determine whether in vitro differences translate into clinically meaningful effects. In particular, post-transfusion recovery and in vivo survival of transfused erythrocytes should be investigated, as these parameters are key determinants of transfusion efficiency. Longer circulation times could potentially reduce transfusion frequency and improve clinical outcomes, especially in vulnerable populations such as preterm infants. Ultimately, prospective clinical trials in neonatal patient cohorts will be necessary to evaluate whether differences in additive solutions and erythrocyte source (cord blood versus adult blood) have a measurable impact on transfusion clinical outcomes such as transfusion requirements and complication rates.

4.1 Limitations

Several limitations of this study should be considered. Due to changes in room conditions during the experimental period, some deep-well plates had to be transferred to a different cold room, which may have introduced additional variability between samples. Furthermore, the experimental setup was limited by a relatively small sample size (n = 6). In addition, the erythrocyte isolation procedure required extensive optimisation and careful handling throughout the study.

Statistical analyses were performed using GraphPad Prism. While the applied REML-based mixed-effects models appropriately account for repeated measurements within donors and are suitable for unbalanced datasets with missing observations, more complex factorial structures were not implemented within a single integrated model framework. Consequently, cord blood and adult erythrocytes were analysed in separate models rather than within a combined analysis. This limits the direct statistical comparability between ontogenetic groups, and differences between groups should therefore be interpreted cautiously.

The preliminary comparison of anticoagulants (heparin vs. CPD-A) was conducted in a single donor using technical replicates only and therefore does not allow statistical

inference or generalisation. Future studies including larger donor cohorts are required to validate these observations.

5 Conclusion

The present study successfully established a reproducible isolation protocol for red blood cells, providing a robust methodological basis for future investigations. Using this approach, the study systematically compared cord blood-derived and adult erythrocytes in seven different additive solutions over four weeks, assessing haemolysis, morphology, lactate accumulation, and glucose consumption as key markers of the storage lesion.

Across most parameters, broadly similar patterns were observed between the two cell populations, indicating that additive solution composition is the primary determinant of storage quality, whereas the effect of cell origin is comparatively minor. In particular, alkaline and phosphate-buffered solutions (AS7, PAGSM, and PAG3M) consistently demonstrated superior preservation, while SAGM induced the most pronounced haemolysis in both groups.

Morphological analysis, however, revealed more pronounced differences between adult and cord blood erythrocytes. In cord blood samples, morphological deterioration was partially uncoupled from haemolysis, suggesting that biochemical and structural storage lesions do not progress in parallel in neonatal cells.

When considering formulation-specific effects, solutions associated with better preservation in cord blood erythrocytes consistently contained higher levels of stabilizing components such as mannitol and adenine. While this may indicate that neonatal erythrocytes are particularly sensitive to overall formulation characteristics, a specific contribution of individual components cannot be confirmed due to the multifactorial differences between additive solutions.

Overall, these findings suggest that while cord blood erythrocytes behave largely similarly to adult erythrocytes with respect to haemolysis and metabolic parameters, they exhibit distinct morphological responses during storage. Importantly, additive

solutions that perform well in adult erythrocytes also appear suitable for cord blood-derived cells, supporting their translational applicability. These results provide a foundation for further optimisation of storage strategies for neonatal transfusion applications.

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7 List of figures

8 List of tables