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DEVELOPMENT OF NEW BLOOD BANKING STRATEGIES FOR PROCESSING AND STORAGE OF RED BLOOD CELL COMPONENTS

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

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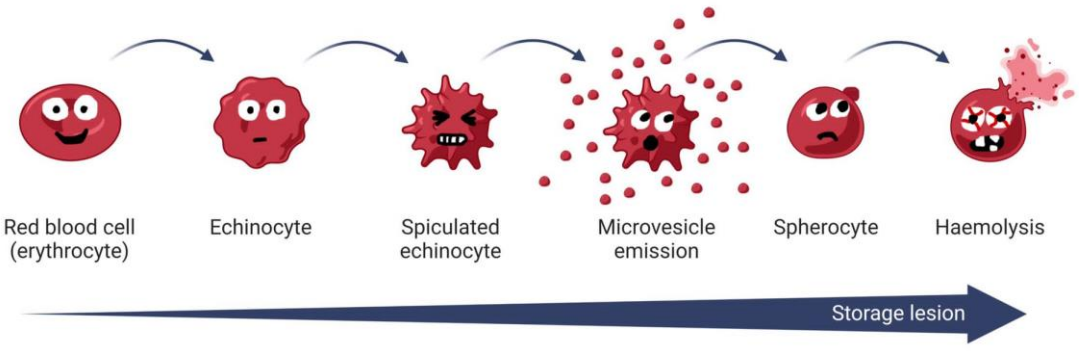
Friends are the family we choose for ourselves. This thesis is dedicated to all my fantastic, fabulous friends who encourage me to be a giant blood nerd, while simultaneously drawing eyes on my red blood cells to provide some perspective…



POPULÄRVETENSKAPLIG SAMMANFATTNING

Röda blodkroppars huvudsakliga uppgift är att se till att kroppens övriga celler också kan göra sitt jobb. Detta sker genom att de röda blodkropparna dels levererar syre som de andra cellerna kan tillverka energi av och dels forslar bort överblivna slaggprodukter. När människor blöder och förlorar röda blodkroppar mår de dåligt och känner sig orkeslösa. Blöder de tillräckligt mycket eller länge överlever de inte ens. Därför är det viktigt att det finns tillräckligt med blod i sjukhusens lager, så att människor som blöder snabbt kan få hjälp.

Blod kan inte tillverkas på konstgjord väg utan doneras av blodgivare. På Blodcentralen delar man upp blodet i beståndsdelar, vilka gemensamt kallas för blodkomponenter. De röda blodkropparna, som är den delen av blodet som i dagligt tal kallas för just ”blod”, får bo i en egen blodpåse av plast. I påsen finns det en lösning som innehåller lite olika godisbitar som de röda blodkropparna kan få energi av och som även gör att de inte klumpar ihop sig. Man skämmer helt enkelt bort dem så att de ska hålla sig så nöjda som möjligt under så långt tid som möjligt, trots att de förvaras i en obekväm plastpåse istället för i sin favoritmänniska. Man kan jämföra plastpåsen med löpbandet på gymmet. På löpbandet är allting frid och fröjd i början, men springer man för länge får man mjölksyra, särskilt om man kör för hårt. Får de röda blodkropparna tillräckligt mycket mjölksyra orkar de inte mer utan ger upp. Förr eller senare kommer de röda blodkropparna alltid att ge upp, men förhoppningsvis inte innan de hunnit komma in i den behövande patienten och hjälpt patienten att må bättre igen. Ju tröttare en röd blodkropp blir och ju mer mjölksyra den får, desto mer dramatiskt börjar den bete sig. Den hissar ”ät-upp-mig”-flaggor, slänger iväg en massa bitar av sitt eget skal och svettas ut olika saltämnen. Dessa ämnen och bitar, vilka alltså skvalpar runt fritt i blodpåsen, kan vara väldigt skadliga för den patient som blir mottagare av blodet. Om den röda blodkroppen inte kliver av löpbandet i tid (och det gör den inte, för den är envis som tusan och har ett pannben av järn) kommer den slutligen att spricka. Då tappar den helt sin förmåga att kunna hjälpa den sjuka patienten.

Plastpåsarna som blodet förvaras i innehåller en så kallad mjukgörare som heter DEHP. Mjukgörare är viktiga för att plasten inte ska gå sönder när man till exempel separerar de röda blodkropparna från resten av blodgivarblodet eller skickar blodpåsarna mellan olika sjukhus. DEHP är de röda blodkropparnas favoritmjukgörare. Jämfört med andra mjukgörare har den nämligen en bonuseffekt: Den fungerar nämligen som ett tillskott som hjälper de röda blodkropparna att hålla sig från att spricka. DEHP har dock visat sig kunna skada könscellerna i manliga bebisråttor. Även om ingen skada har kunnat ses i människor under de 70 år DEHP har använts, har Europakommissionen nyligen tagit ett beslut om att användningen av DEHP ska förbjudas. Alltså måste en ny mjukgörare hittas, som fungerar lika bra som DEHP för både blodpåsens och de röda blodkropparnas kvalitet. Hittills har det varit väldigt klurigt att hitta en mjukgörare som klarar av det sistnämnda – att boosta de röda blodkropparna. Nu har det dock tagits fram en möjlig kandidat: DEHT. Nästan samma namn som DEHP alltså, men snällt både mot råttor, människor och miljö! I **Studie II** och **III** testade jag och mitt team hur de röda blodkropparna mådde av att förvaras i blodpåsar gjorda av DEHT. I **Studie II** fick de röda

blodkropparna ideala förutsättningar på ”gymmet”, och i **Studie III** utmanade vi dem genom att utsätta dem för extrem stress genom röntgenbestrålning – något av det värsta de röda blodkropparna vet! När röda blodkroppar bestrålas kastar många av dem in handduken direkt. Av de röda blodkroppar som inte spricker, får många stora hål i sitt skal så att ännu fler läskiga ämnen kan läcka ut i blodpåsen. På grund av dessa effekter brukar man förkorta hållbarheten på bestrålat blod så att det fortfarande är säkert för patienten som ska få det. Ett förkortat bäst-före-datum gör dock att risken ökar att det blir brist på blod i sjukhusens lager.

Studieresultaten visade glädjande nog att DEHT höll måttet riktigt bra! De röda blodkropparna var aningen mindre nöjda, men på det stora hela var deras kvaliteten ändå klart godkänd. Det mesta av missnöjet gick dessutom att korrigera om man ändrade en aning på sammansättningen av godis som de röda blodkropparna får med sig i sin förvaringspåse från första början.

Det är viktigt att testa att bestråla blod i DEHT-påsar, eftersom man ibland behöver kunna göra det i den dagliga verksamheten. Separationen av röda blodkroppar från blodgivarblodet är nämligen inte helt hundraprocentig. Bland de röda blodkropparna gömmer sig alltid även några vita blodkroppar. De vita blodkropparna är kroppens immunförsvar och uppfattar allting som inte är eget som främmande. Om vita blodkroppar från blodgivaren åker snålskjuts på de röda blodkropparna in i en patient som blöder men har ett normalfungerande eget immunförsvar, händer ingenting. Patientens eget immunförsvar kommer enkelt att oskadliggöra snyltgästerna. Om patienten som blöder däremot har ett dåligt eget immunförsvar, t.ex. som biverkning av cancerbehandling eller för att patienten i fråga är en bebis som har hunnit utveckla ett starkt eget immunförsvar än, finns det risk för att de snålskjutsåkande vita blodkropparna uppfattar hela patienten som ett ”främmande föremål” och attackerar patienten, inte sällan med dödlig utgång. Om man därför bestrålar blod med röntgenstrålning paralyserar man de vita blodkropparna så att de inte kan attackera patienten. Eftersom röntgenstrålning trots allt är ganska omilt även mot de röda blodkropparna (dock inte i närheten så elakt som mot de vita), bestrålar man blodet enbart i de fall då patientens eget immunförsvar inte är fullt fungerande.

Blod kan tvättas också. Bland de röda blodkropparna gömmer det sig nämligen även lite blodplasma av samma anledning som det gömmer sig vita blodkroppar. Människor kan vara allergiska mot ämnen i plasman, precis på samma sätt som de kan ha allergi mot pollen eller jordnötter. Om dessa människor behöver blod brukar man tvätta bort blodplasman för att inte råka framkalla en allergisk reaktion. Tvätten går till precis som det låter: Man stoppar blodet i en tvättmaskin som är anpassad för blod istället för kläder och så trycker man på start och tvättar bort plasman. Det är väldigt effektivt, men de röda blodkropparna blir lite stressade även av att tvättas och svarar även i detta fall med att spricka och åbäka sig i högre grad än om man lämnar dem ifred. Därför kortar man ned bäst-före-datumet även på tvättat blod.

Normal hållbarhet för blod i Sverige är 6 veckor. Om man bestrålar eller tvättar blodet blir hållbarheten istället mellan 2 och 4 veckor. I **Studie IV** provade jag och teamet en alternativ teknologi till bestrålning och tvättning som sägs vara snällare mot de röda blodkropparna men fortfarande lika effektiv för att oskadliggöra de vita blodkropparna. Teknologin kallas patogenreducering (ja, extremt krångligt namn, men man behöver inte förstå det, endast

acceptera det!). Patogenreducering är egentligen utvecklat för att inaktivera andra typer av snyltgäster i blodet så som virus, bakterier och parasiter, men en bonuseffekt är att det även är effektivt mot de vita blodkropparna. Den teknologi som användes i studien innehåller dessutom ett moment som potentiellt skulle kunna fungera som tvätt.

Tanken med studien var att dels att testa om blodet mår tillräckligt bra efter patogenreducering för att dess ursprungliga hållbarhet, 6 veckor, ska kunna behållas. Jag ville även kontrollera hur effektivt blodplasman tvättades bort. Masterplanen var att se om det i framtiden är möjligt att ersätta både bestrålat och tvättat blod med en gemensam blodkomponent, patogenreducerat blod. Våra studieresultat visade att detta var möjligt, även om man möjligtvis skulle vilja mickla en aning med inställningarna för tvätten för att få en ännu renare slutprodukt.

Sist – och faktiskt minst, rent fysiskt – är **Studie I**, där jag och mitt team uppfann en teknik för att kunna frysa bäbisportioner av blod. Anledningen till att man ibland fryser blod är att man vill kunna spara det mycket längre än de normala 6 veckorna. Det finns nämligen en del undergrupper av blod som är väldigt sällsynta och extremt svåra att få tag på när en patient som behöver en sådan undergrupp blöder. Ibland finns den närmsta blodpåsen i Brasilien! Fryst blod kan förvaras upp till 30 år i Sverige, vilket är en enorm förbättring mot 6 veckor. När en blodgivare med en sällsynt undergrupp dyker upp fryser man därför gärna denna blodgivares blod. Man kan dock inte lägga en blodpåse direkt i frysen. Då spricker blodet, eftersom vätskan inne i de röda blodkropparna bildar iskristaller. Därför använder man en ganska avancerad teknik som går ut på att överlista iskristallbildningen. De röda blodkropparna är inte överdrivet entusiastiska över behandlingen och endast ca 80 % av dem klarar sig helskinnade genom processen utan att spricka. 80 % är dock fortfarande enormt mycket bättre än att inte ha något matchande blod alls till en patient.

Bebisar behöver oftast väldigt små portioner blod åt gången. Därför är det synd att tina en hel påse med fryst, sällsynt blod och behöva slänga bort den del av blodet som inte går åt, särskilt om bebisen behöver mer blod några veckor senare. Tidigare har det inte funnits något sätt att frysa och tina bebisportioner av blod. I **Studie I** uppfann vi en metod för att göra det genom att lägga till en manuell tvist till den klassiska tvättmaskinstekniken (vilken även kan användas för frysning). Studieresultaten visade att bebisportionerna av blod mådde minst lika bra som en normalportion, trots att vi egentligen utsatte blodet som var uppdelat i bebisportioner för mer stress totalt, vilket de röda blodkropparna borde ha blivit arga över. Detta var oväntat, men väldigt positivt!

Sammanfattningsvis undersökte jag och mitt team fyra olika, nya sätt att behandla eller förvara röda blodkroppar, vilka tillsammans kan bidra till att öka tillgängligheten av blod och därmed öka säkerheten för patienterna. En ökad tillgänglighet och ett totalt sett mindre sårbart blodlager är extra viktigt eftersom antalet blodgivare minskar i samhället till följd av att det inte föds lika många människor som tidigare. Ingen kan magiskt trolla fram mer blod, men vi kan behandla det vi har smartare!

POPULAR SCIENCE SUMMARY

The main task of the red blood cells is to serve the other cells in the human body so that they also can do their job. The red blood cells facilitate this by delivering oxygen that the other cells can use to produce energy and also by removing garbage from the energy production. When people bleed and lose red blood cells, they feel bad and get very tired. If the bleeding isn't stopped, they eventually die. Therefore, it is important to ensure that there are sufficient inventories of red blood cells at the hospitals, so that a patient who bleeds can receive help quickly.

It is not possible to engineer blood. Therefore, it is donated by blood donors. At the blood establishment, the donated blood is divided into its different parts, called blood components. The part that consists of red blood cells, often simply referred to as "blood" in everyday language, is stored in its own blood bag made of plastics. The bag contains a solution made of different pieces of 'candy' that energise the red blood cells and also make sure that they don't stick together. Basically, we try to spoil the red blood cells to keep them as happy as possible for as long time as possible, to distract them from remembering that they are stored in a plastic bag instead of in their favourite human being. The bag can be compared to the treadmill at the gym. On the treadmill, everything is fine in the beginning, but if you run too fast or too long, the muscles of your legs will start filling up with lactic acid. If the red blood cells produce too much lactic acid, they get tired and give up. Sooner or later, they will always give up, but ideally, not before they have been delivered to the patient who needs them and helped the patient to feel better again. The more tired a red blood cell becomes and the more lactic acid it produces, the more dramatically it behaves. It hauls don't-eat-me flags, it throws around small pieces of its own shell, and it sweats out different salt compounds. These pieces and compounds can be very dangerous to the patient who receives the blood. If the red blood cell doesn't step off the treadmill in time (and it never does, it is way too stubborn), it will finally burst. When it bursts, it loses all ability to help the sick patient.

The blood bags that the red blood cells are stored in contain a so-called plasticizer named DEHP. Plasticizers are essential for making the blood bags soft and flexible and easy to handle without breaking, for instance during the separation of the red blood cells from the rest of the donor blood, or during transport between different hospitals. DEHP is the red blood cells' favourite plasticizer. Compared to other plasticizers, it comes with a bonus effect: It works as an add-on that strengthens the red blood cells so they don't burst as easily. However, DEHP is suspected to interfere with the hormones of male baby rats. Even though no such damage has been detected in human beings during 70 years of usage, the European Commission has recently decided to prohibit the use of DEHP. In other words, a new plasticizer needs to be found, that is as good for the blood bag properties as for the quality of the red blood cells. So far, it has been very difficult to find a plasticizer that can ensure the last part – boosting the red blood cells. A possible candidate has recently been suggested: DEHT. Almost the same name as DEHP, but nice to both rats, humans and the environment! In **Papers II and III**, my team and I examined the well-being of red blood cells stored in blood bags made of DEHT. In

Paper II, the red blood cells were given ideal storage conditions, whereas in **Paper III**, we challenged the red blood cells by exposing them to severe stress through X-ray irradiation – one of the worst things the red blood cells know! When red blood cells are irradiated, many of them give up instantly. Of those who fight back, many still get their shell punctured in multiple places, which means that an even higher amount of dangerous substances leaks out into their surrounding liquid in the blood bag. Because of these effects, the shelf-life of irradiated blood is usually shortened to make sure that the blood is still safe enough for the patient. However, shortened shelf-life means that the risk of an overall shortage of blood at the hospitals increases.

Our study showed that DEHT worked better than we had dared to hope for! The red blood cells were not quite as happy as when they were stored in DEHP bags, but overall, their quality was still satisfying. In addition, most of the red blood cells' complaints could be silenced by slightly upgrading the "candy solution" that the red blood cells are mixed with in the blood bag.

It is important to test irradiating red blood cells stored in DEHT bags, since blood sometimes needs to be irradiated in ordinary situations. The separation of red blood cells from the rest of the donor blood is not 100 %. A few white blood cells are always hiding amongst the red blood cells. The white blood cells are the body's so-called immune system – a defence against anything foreign. The white blood cells are alerted by everything they encounter that doesn't look like themselves. If white blood cells from the donor follow the red blood cells into a patient who bleeds but have a functioning immune system, nothing happens. The immune system of the patient will easily take down the trespassers. However, if the bleeding patient has a weakened immune system because of cancer treatment or because the patient is a baby that has not yet developed a full defence, there is a risk that the white blood cells consider the entire patient a "foreign object" and attacks the patient, not seldom with a fatal outcome. To prevent such a scenario, the blood can be X-ray irradiated. Irradiation paralyses the white blood cells so that they will not be able to attack the patient. Since X-ray irradiation is quite harsh to the red blood cells (though not nearly as brutal as it is to the white blood cells), blood is irradiated only when the defence of the patient is weakened.

Blood can also be washed. Among the red blood cells, there is also some blood plasma hiding, for the same reason as there are some white blood cells lurking around. People can be allergic to plasma, the same way as they can be allergic to peanuts or pollen. If allergic people need blood, it is common to wash away the plasma first, to not risk that the patient gets an allergic reaction. Washing of blood works the same way as washing of clothes: You put the blood in a washing machine for blood, press the start button, and wash away the plasma. It's a very effective procedure, but the red blood cells get stressed out from being washed, and answer also in this case by bursting or complaining much more than if they had been left alone in peace. Therefore, also the shelf-life of washed cells is shortened.

Normal shelf-life of red blood cells in Sweden is 6 weeks. If the blood is irradiated or washed, the shelf-life is shortened to between 2 and 4 weeks. In **Paper IV**, the team and I tried an alternative technology to irradiation and washing that is supposed to be more gentle to the red blood cells but still as efficient in rendering the white blood cells harmless. The technology is

called pathogen reduction (yes, extremely complicated word, but there's no need to understand it, just to accept it!). Pathogen reduction was originally developed to inactivate other kinds of blood trespassers such as viruses, bacteria and parasites, but a bonus is that it is also effective against white blood cells. The technology that was used in this study additionally contains a step that potentially could be used as washing.

The idea of the study was to test if the blood had a good enough quality after pathogen reduction to motivate that its original 6-weeks shelf-life could be kept. I also wanted to check how effectively the plasma was washed away. The master plan was to test whether it in the future will be possible to replace both irradiated and washed blood with a common component, pathogen reduced blood. Our study results showed that this was possible, even if it would be tempting to play a little with the settings of the washing step to get an even cleaner final product.

Last – and actually least, physically – is **Paper I**, where my team and I invented a technique to be able to freeze baby portions of blood. To be able to stock-keep blood for much longer time than the normal 6 weeks, blood is sometimes frozen. There are some sub-types of red blood cells that are extremely rare and difficult to quickly get hold of when a patient that needs such a sub-type is bleeding. Sometimes, the nearest blood bag is located in Brazil! Blood can be stored frozen for up to 30 years in Sweden. This is of course a huge improvement compared to 6 weeks. Therefore, blood from donors who have rare blood types are often frozen. It is not possible to put a bag of blood straight into the freezer. If it is done, the red blood cells burst, because the liquid inside the red blood cells forms ice crystals. Therefore, an advanced technique is used that is based on outsmarting the ice crystal formation. The red blood cells are not overly enthusiastic about the treatment, and only about 80 % of them makes it through the process without bursting. However, 80 % is still much better than not having any matching blood in stock to a bleeding patient.

Babies often only need very small portions of blood at a time. Therefore, it is a bit sad to thaw an entire bag of frozen, rare blood and throw away the largest part of it, especially if the baby needs more blood a few weeks later. Previously, a method for freezing and thawing baby portions of blood has not existed. In **Paper I**, we invented such a method through adding a manual twist to the classical washing method (the machine can be used also for freezing). The study results showed that the baby portions had at least as high quality as a normal portion, even though we stressed the blood divided into baby portions more, which the red blood cells should have been angry about. This was unexpected, but very positive!

To summarise, my team and I investigated four new, different ways of treating or storing red blood cells, which together can contribute to increasing the availability of blood and thereby increase the safety for the patients. An increased availability and overall less vulnerable inventory of blood is extra important considering that the number of blood donors are sinking as a consequence of lower birth rates compared to previously. No one can make more blood magically appear, but we can treat the blood we have got in a smarter way!

ABSTRACT

The field of red blood cell (RBC) components has been quite uneventful during the last few decades, save for the experimental exploration of alternative additive solution compositions. The reason is likely that the RBC quality has been good enough and the shelf-life of conventional RBC concentrates (RCC) has been long enough to not really motivate the workload that any dramatic changes would implicate.

However, the concern for future blood supply shortage is now growing in Europe. It is incited by multiple factors; perhaps foremost, the oncoming ban of the blood bag plasticizer di(2-ethylhexyl) phthalate (DEHP). DEHP prolongs the RBC's lifespan during blood bank storage by stabilising the RBC membrane, and removal of DEHP has been linked to unacceptable haemolysis levels. For a long time, it has been a challenge to find a replacement to DEHP that does not compromise the RBC quality or RCC shelf-life. Concomitantly with the imminent non-DEHP transition, new regulatory frameworks call for better blood supply contingency and preparedness for emergency situations, and the healthcare community encourages more individualised treatment therapies. A contra-indication to all of these proceedings is that the donor population is decreasing due to smaller birth cohorts, simultaneously as the number of patients needing transfusion therapy is increasing due to longer life expectancy and more successful treatment of diseases.

The four studies of this theses have focused on different ways to approach the same aim of preserving or even improving the RBC quality, limit the outdating frequency and, ultimately, ensure a safe and sufficient blood supply.

Whole blood was processed into RCCs, which were further treated to fit the objectives of each respective study. In **Paper I**, a method was developed to cryopreserve split RCCs, by combining the ACP 215 automated cell processor with a subsequent manual centrifugation step. In **Papers II and III**, a four-armed study compared storage or RCCs in DEHP to a suggested substitute plasticizer, di(2-ethylhexyl) terephthalate (DEHT), combined with either of the two additive solutions saline-adenine-glucose-mannitol (SAGM) or phosphate-adenine-glucose-guanosine-saline-mannitol (PAGGSM). **Paper II** addressed ideal storage conditions whereas **Paper III** exposed the RBCs to extreme oxidative stress in the form of X-ray irradiation. Irradiated and ACP 215-washed RCCs were then compared to RCCs pathogen reduced with the Intercept blood system in **Paper IV**. All studies assessed the RBC quality after intervention and during subsequent storage by utilising a battery of analyses that together determined the RBC storage lesion, i.e. the RBCs metabolic, morphologic and oxidative status as well as the direct preservation of its membrane.

Paper I demonstrated that it was possible to successfully cryopreserve split RCCs without negatively impacting the quality of the final component compared to traditional, non-split cryopreserved RCCs. In fact, the levels of haemolysis and extracellular potassium ions (K^+) were both lower than in non-split cryopreserved RCCs, which emphasised the compatibility of split cryopreserved RCCs in a paediatric transfusion setting. The quality of all the split RCCs

was very even, demonstrating the robustness and reproducibility of the protocol, which is essential for everyday blood banking. **Papers II and III** verified DEHT as a strong potential substitute plasticizer to DEHP, even though the RBC membrane integrity was slightly impaired, foremost during SAGM storage. Irradiation expectedly introduced additional membrane damage, but satisfactory, the haemolysis was still well within the allowed margin. Independent of the level of stress exposure, the results suggested that if a transition to PAGGSM is adopted at the same time as a new plasticizer is implemented, any deleterious effects of the DEHP removal can be strongly mitigated. The two papers also confirmed that the RBC metabolism was unaffected by the switch of plasticizer. **Paper IV** demonstrated that, in addition to increased safety in terms of transfusion-transmitted infectious diseases, pathogen reduction is a promising future option to both irradiation and washing of blood components. Pathogen reduced RCCs exhibited membrane preservation similar to conventional RCCs and far superior to irradiated and automated-washed RCCs, where shelf-life reduction is a necessary adverse measure. With pathogen reduction, the shelf-life of conventional RCCs in Sweden, 42 days, would still be feasible. In addition, pathogen reduced RCCs implicated better ATP preservation, which may be beneficial for the RBC *in vivo* survival; however, at the expense of 2,3-DPG. Considering the washing-specific parameters, implying efficacy of plasma reduction, minor adjustments of the centrifugation protocol would still be desirable.

In conclusion, this thesis explores the multiple pathways of RBC processing in order to develop or refine RBC components or RBC storage. The purpose behind this is to propose a way to adapt to the growing urge of ensuring availability of a blood supply fit for all essential transfusions to all categories of patients. In four individual papers, this thesis shows the deleterious effects of RBC cell stress, but also proposes a joint common approach to mitigate it or, in some cases, even improve the components further compared to the previous standard. Hopefully, this innovative view on classical RBC components, along with the presentation of a satisfactory plasticizer option, may inspire to the introduction of a number of measures that can contribute to decreased RCC wastage or prevention of shelf-life reduction. These are two key attributes in increasing patient safety.

LIST OF SCIENTIFIC PAPERS

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A novel protocol for cryopreservation of paediatric red blood cell units allows increased availability of rare blood types
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Non-phthalate plasticizer DEHT preserves adequate blood component quality during storage in PVC blood bags
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III. **Larsson L**, Ohlsson S, Derving J, Diedrich B, Sandgren P, Larsson S, Uhlin M
DEHT is a suitable plasticizer option for phthalate-free storage of irradiated red blood cells
Vox Sang. 2022 Feb;117(2):193–200. doi: 10.1111/vox.13177. Epub 2021 Jul 15.

IV. **Larsson L**, Ohlsson S, Neimert Andersson T, Watz E, Larsson S, Sandgren P, Uhlin M
Pathogen reduced red blood cells as an alternative to irradiated and washed red blood cells with future potential for storage up to 42 days
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SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

V. Gulliksson H, Meinke S, Ravizza A, **Larsson L**, Höglund P
Storage of red blood cells in a novel polyolefin blood container: a pilot in vitro study
Vox Sang. 2017 Jan;112(1):33–39. doi: 10.1111/vox.12472. Epub 2016 Dec 21.

CONTENTS

LIST OF ABBREVIATIONS

2,3-DPG	2,3-diphosphoglycerate
AABB	Association for the Advancement of Blood & Biotherapies
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BTHC	n-Butyryl tri-n-hexyl citrate
CPD	Citrate-phosphate-dextrose
DEHP	Di(2-ethylhexyl) phthalate
DEHT	Di(2-ethylhexyl) terephthalate
DINCH	1,2-Cyclohexanedicarboxylic acid, diisononyl ester
EDQM	European Directorate for the Quality of Medicines & HealthCare
EVA	Ethylene-vinyl acetate
GSH	Glutathione,
GSSG	Glutathione disulphide
GTP	Guanosine triphosphate
Hb	Haemoglobin
Hct	Haematocrit
MDR	Medical Device Regulation
MEHP	Mono-2-ethylhexyl phthalate
NADH, NAD^+	Nicotinamide adenine dinucleotide
NADPH, $NADP^+$	Nicotinamide adenine dinucleotide phosphate
PAGGGM	Phosphate-adenine-glucose-guanosine-gluconate-mannitol
PAGGSM	Phosphate-adenine-glucose-guanosine-saline-mannitol
PR	Pathogen reduction, pathogen reduced
PVC	Polyvinyl chloride
RBC	Red blood cell
RCC	Red blood cell concentrate
RMV	Red blood cell microvesicle
ROS	Reactive oxygen species
RT	Room temperature
SAGM	Saline-adenine-glucose-mannitol
TA-GvHD	Transfusion-associated graft-versus-host disease
TRALI	Transfusion-related acute lung injury
UV	Ultraviolet
WB	Whole blood

1 INTRODUCTION

1.1 BACKGROUND

The principal task of red blood cells (RBC) is to deliver oxygen to the body's tissues and organs via the vascular system. Transfusion of RBCs is indicated to compensate a deficit caused by critical bleeding or to correct anaemic conditions, in order to increase the oxygen-carrying capacity and avoid detrimental effects caused by oxygen debt[1]. RBCs have been regularly separated from whole blood (WB) and stored as RBC concentrates (RCC) since the middle of the 20th century, when polyvinyl chloride (PVC) blood bags were introduced as storage containers. Prior to that, blood was collected and stored in glass bottles. The use of PVC plastic bags, linked together with a transfer tube system in a bacteriologically closed system, is considered the springboard to modern everyday-practice of blood component therapy[2]. Transfusion of blood components, as opposed to unseparated WB, allows for a tailored therapy focusing on correction of specific deficiencies or conditions. Through component therapy, unwarranted transfusions can be avoided, which reduces both the overall risk of blood transfusion and component wastage; the latter of which is imperative for the sustainability of a sufficient blood supply. In addition, separation into different blood components allows for optimised storage time and conditions, and thereby function and viability, for each separated component ***(Figure 1)***.

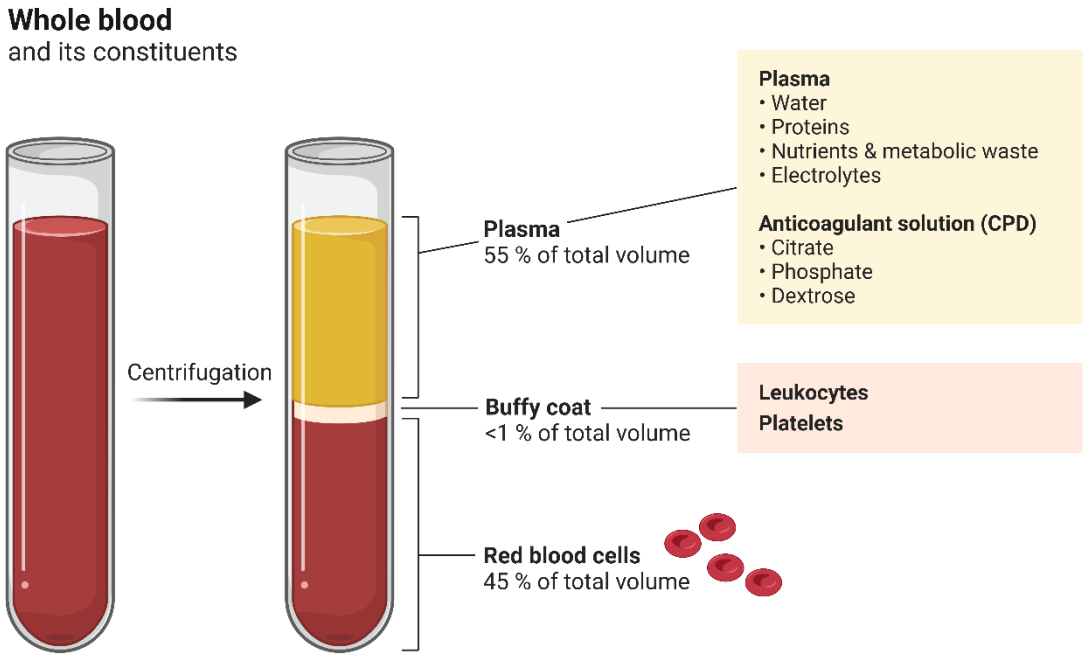


Figure 1. *The different constituents of whole blood, separated in density order. Approximately 45 % of the total volume constitutes of red blood cells before addition of anticoagulant solution.*

Since this early development, methods, materials and processes have been refined, but the essence of RBC processing has remained the same: RBCs are packed and separated through centrifugation, after which they are resuspended in additive solution to provide good storage conditions, and kept in refrigerated conditions until transfusion. Over the years, the composition of the additive solution has been explored to optimise the RBC metabolism and prolong shelf-life, but overall, no dramatic changes have been seen for a conventional RCC over the past few decades.

After a long period of stability, the RBC components are now facing a series of challenges. Concerns about toxicity regarding the blood bag plasticizer di(2-ethylhexyl) phthalate (DEHP)[3], both to patients and the environment, is enforcing new blood bag materials onto the market, which are expected to impact the RBC storage quality[4,5]. Increased global temperature is threatening to introduce tropical blood-borne parasites and diseases in expanded geographical areas. These include colder places like Sweden, which up until now have been considered suboptimal to their survival. Increased migration throughout the world and easier access to travel enhance this risk, and furthermore facilitate a growing mismatch in blood types between donors and recipients, which puts pressure on a blood supply that is already challenged by a general blood donor population decrease and a longer life expectancy[6-8].

Simultaneously, the need to identify the blood supply as a critical healthcare priority has been recognised on a regulatory level. The European Commission has adapted a proposal for an updated legal framework for blood, tissues and cells, which urges all European Union (EU) countries to implement strategies to be able to, when faced with an emergency situation, ensure a safe and adequate supply of blood for all essential transfusions[9]. The need for effective contingency and emergency planning has been actualised by recent events such as the COVID-19 pandemic, frequent extreme weather events, terrorism and armed conflicts[10,11].

To meet the demand of ensuring the blood supply in a time where its quality and availability is threatened, it is necessary to act pro-actively and explore new angles of the RBC processing and storage strategies.

1.2 PROCESSING OF RED BLOOD CELLS

RCCs can be derived from WB or via apheresis technology, in which the RBCs are singled out directly during donation. The majority (> 99 %) of the RCCs collected in the Council of Europe member states come from WB separation into blood components[12].

The WB separation process is based on two key steps: centrifugation, where the different components are separated according to density, and extraction, in which the centrifugal layers are separated through transfer to isolated bags. For RBCs, the process continues with addition of additive solution and leukoreduction through filtration ***(Figure 2).***

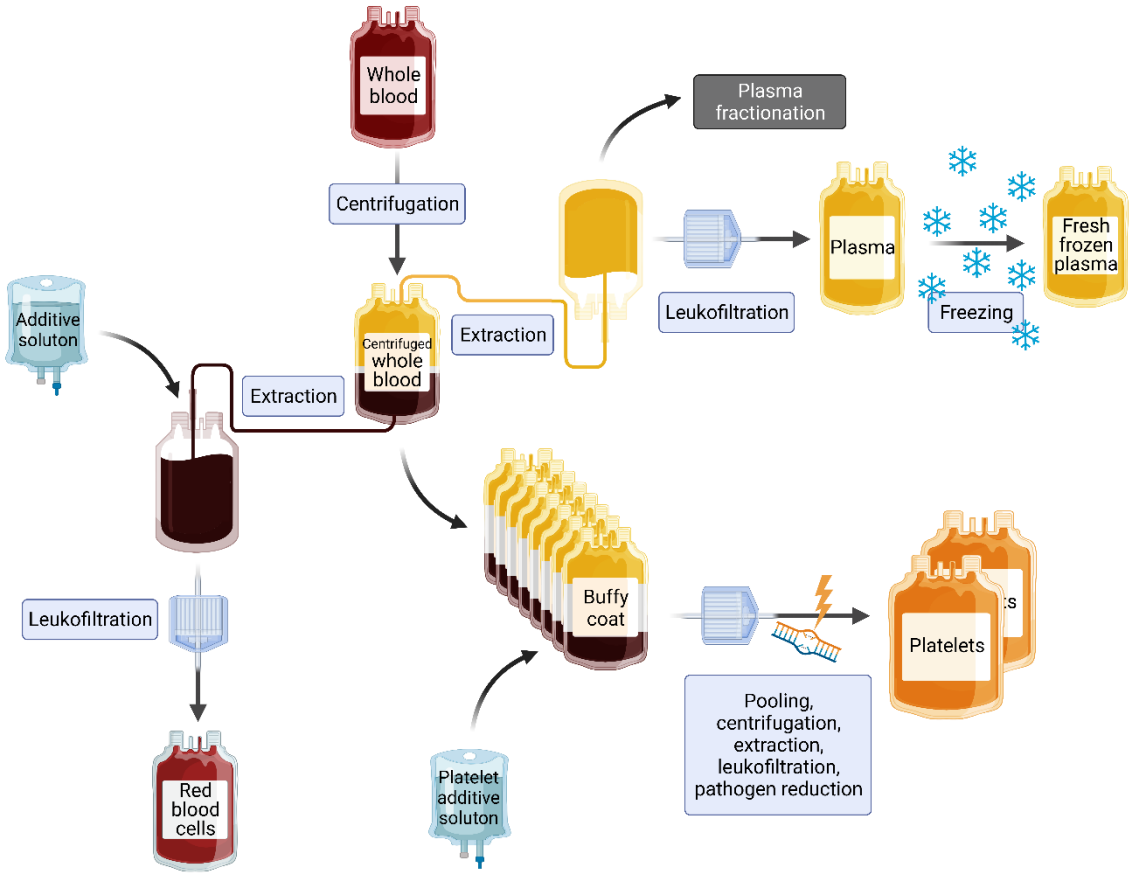


Figure 2. *Processing of whole blood into the three primary blood components: red blood cell concentrates, plasma and platelet concentrates. The figure illustrates the processing strategy of the blood establishment of Karolinska University Hospital, Stockholm, Sweden.*

1.2.1 Processing strategies

A number of key processing elements affect the RBC quality. It is important to be aware of their respective impact on the blood components when choosing processing strategy:

- **Time and temperature:** WB can be separated either on the collection day or after overnight storage in room temperature (RT). Although up to 24 hours delay between WB collection and cold storage (2–6 °C) of the processed RCCs is accepted according to European guidelines (established by the European Directorate for the Quality of Medicines & HealthCare, EDQM[13]), the "the faster, the better" proverb applies. By lowering the temperature of the RBCs, their metabolism is impeded, which is associated with reduced storage lesion, as explained in more detail in **Chapter 1.3.2.** From this point of view, separation on the collection day is preferable, but for logistical reasons, many blood establishments choose overnight storage of the WB before processing start[14].

  A complementary way of reducing the progression of the RBC storage lesion is the practice of actively cooling down the WB from 37 °C to RT immediately after

collection. By using this measure, the rate of the RBC glycolysis can be reduced by half[15,16].

- **Buffy coat removal and leukoreduction:** There are two main approaches for leukoreduction of RBCs. In situations where buffy coats (containing platelets, leukocytes and residual plasma/RBCs) are further utilised for platelet processing, the RBCs are leukoreduced by filtration after they have been separated from the other components and mixed with additive solution ***(Figure 2)***. If platelet concentrates are not manufactured as part of the overall processing strategy, leukoreduction of the RBCs is performed as part of a WB leukofiltration process before the WB centrifugation. It has been demonstrated that RBC leukofiltration is superior to WB filtration in order to mitigate increased generation of haemolysis[17,18]. The downside with the RBC leukofiltration strategy is that a small amount of RBCs (16–18 mL; Karolinska blood establishment routine processing) is lost in the buffy coat. Except for the obvious disadvantage of losing RBCs for transfusion[14], this loss also has an impact on particular, volume-dependent secondary processing steps, such as washing of RCCs with automated equipment[19].

- **Centrifugation:** Extensive mechanical stress is linked to increased levels of haemolysis; rupture of the RBC membrane. Therefore, WB is rarely centrifuged at a g-force exceeding 5000 × g. Although many blood establishments centrifuge WB at this speed with satisfactory RCC haemolysis levels at storage end, reducing the speed could potentially reduce the haemolysis further, especially in situations where the RCCs are exposed to subsequent stress through the processing of secondary components or storage in non-DEHP blood bag material, as described further in **Chapter 1.3.2.6.**

1.2.2 Leukoreduction

Pre-storage leukoreduction, either via filtration or corresponding reduction technology during apheresis collection, has several advantages for the RBC component as well as for patient safety. Reduction of the number of leukocytes to $< 1 \times 10^6$ leukocytes per transfusion unit (maximum count to qualify as a leukoreduced component[13]), substantially reduces the risk of introducing adverse transfusion reactions, immunisation against leukocyte antigens, transfusion-associated graft-versus-host disease (TA-GvHD) and transmission of cytomegalovirus[20,21]. In addition, leukoreduction also decreases the rate of haemolysis during storage, as leukocytes release proteases and generate reactive oxygen species (ROS) when they are degraded[17,18]. Because of these advantages, pre-storage leukoreduction is nowadays routine practice in almost all European countries12.

1.2.3 Whole blood anticoagulant solutions

After collection, the RBCs are supported by both the plasma and a WB anticoagulant solution. The plasma provides nutrients and buffering via dextrose, phosphate and bicarbonate. The anticoagulant solution is added in the ratio of approximately 1:7 to the WB[22] (equalling 63 mL anticoagulant solution to 450 mL WB). It most commonly constitutes of citrate-phosphate-

dextrose (CPD), even though several successful attempts have been made to refine its composition[23-26]. In CPD, citrate provides the anticoagulant properties, whereas dextrose and phosphate facilitate metabolic functions, not only for RBCs, but also for platelets.

1.2.4 Additive solutions

As the WB anticoagulant solution is lost to the plasma fraction during processing, an additive solution, optimised for RBC storage, is added to the RBCs during or immediately after extraction, depending on the nature of the extraction equipment. The ratio of additive solution to RBCs is approximately 1:1.5 to 1:2; 100 mL additive solution is added to the RBC fraction obtained from 450 mL WB (usually 150–200 mL packed RBCs), giving the RCC a final volume of approximately 240–300 mL and haematocrit (volume percentage of RBCs in the RCC) between 50 and 70 %. There are several commercially available additive solutions. All are designed with the purpose to provide nutrients and substances that mitigate storage lesion, and ensure optimal haematocrit.

In Europe, saline-adenine-glucose-mannitol (SAGM) is currently the predominant choice of additive solution. The functions of the different ingredients are as follows:

- **Saline (sodium chloride):** dilutes the packed RBCs to an adequate haematocrit that is a compromise between ensuring an adequate blood flow and preventing patient hyperhydration and disadvantageous osmotic properties.
- **Adenine:** building block for adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NADH); central molecules/ions of the RBC metabolism.
- **Glucose (dextrose):** starting substance in the glycolysis; the almost exclusive metabolic pathway for the RBC.
- **Mannitol:** antioxidant and membrane stabiliser that protects the RBC membrane from haemolysis.

SAGM enables RCC storage up to 42 days. An alternative to SAGM is the next-generation additive solution phosphate-adenine-glucose-guanosine-saline-mannitol (PAGGSM), licensed for corresponding storage up to 49 days in certain European countries. In addition to the ingredients shared with SAGM, the added phosphate and guanosine hold the following functions:

- **Phosphate:** provides phosphate for ATP and GTP (guanosine triphosphate) generation, and in addition, some limited buffering capacity.
- **Guanosine:** building block in GTP, which can replace or complement ATP in some phosphorylation steps of the glycolysis.

Several studies, including **Papers III and IV**, have demonstrated lower haemolysis and reduced storage lesion during storage in PAGGSM when compared to SAGM; both in conventional PVC-DEHP blood bags and during storage in alternative plasticisers such as di(2-ethylhexyl) terephthalate (DEHT) and 1,2-Cyclohexanedicarboxylic acid, diisononyl ester

(DINCH)[27-29]. PAGGSM is therefore a highly interesting choice of additive solution when the DEHP ban comes into effect.

Additive solutions and their relation to pH are described in more detail in **Chapter 1.3.2.5.**

1.2.5 Secondary processing

A conventional RCC consists of packed RBCs and additive solution. The volume varies slightly between RCCs depending on the haematocrit of the donor. There is also a small part of residual plasma (approximately 10–30 mL) and an even smaller fraction of CPD that remains because the centrifugation processing step does not yield 100 % separation. Furthermore, as previously mentioned, residual leukocytes can be present up to 1×10^6 per RCC[13].

With time, the RCC also contains increasing levels of RBC debris, released as a consequence of storage-related changes. Such debris consists of, among other degradation products, free haemoglobin and iron ions, free potassium ions and vesicles emitted from the RBC membrane[30,31]. In rare cases, primarily if routines for donor selection, donation or processing have been inaccurately followed, the RCC can also contain blood-borne pathogens such as virus, bacteria or parasites.

For most patients, the contents and characteristics of a conventional RCC is considered safe enough, and do not pose an unreasonable risk at transfusion. However, specific patients or patient categories may require further tailored adaption or optimisation of the RCC, referred to as secondary processing.

1.2.5.1 Cryopreserved red blood cells

The definition of a 'rare blood type' varies between different geographical parts of the world and depends on the ethnicity of the population[32]. Regardless of the lack of a standardised prevalence number, complications arise when a patient lacks a high-incidence antigen and has formed alloantibodies against it.

To increase the availability of RBCs of rare blood types, the RBCs can be stored cryopreserved instead of refrigerated. With a controlled, standardised freezing and thawing process, RBCs can be kept in frozen condition for at least 10 years[33] (30 years is applied in Sweden), an enormous shelf-life extension compared to the standard 35–42 days.

There are two general approaches for cryopreservation, referred to as the high- and the low-glycerol method. Glycerol serves as cryoprotectant in both. The high-glycerol method uses 40 % weight/volume glycerol, a slow freezing rate (1–3 °C per minute) and allows storage of the frozen RCCs in common mechanical −60–80 °C freezers. The low-glycerol method is based on 20 % weight/volume glycerol and demands plunge freezing in (−150 °C) liquid nitrogen[34,35]. Because of the extreme storage temperature, the low-glycerol method is not compatible with PVC tubes, essential for sterile docking. The high-glycerol method protects the cells better and is associated with less haemolysis than the low-glycerol method, but removing the larger amount of glycerol is more cumbersome[34]. The challenge of glycerol

removal has partially been resolved through introduction of the ACP 215 Automated Cell Processor (Haemonetics Corp., Braintree, MA, USA); an automated, closed-system device for glycerolisation, deglycerolisation and washing[36]. The ACP 215 uses pre-decided flow rates and a standardised shaking mechanism to ensure optimal protection from ice crystal formation[37], during both glycerolisation and the multi-step deglycerolisation/washing process.

The possibility to keep the system bacteriologically closed enables after-thawing shelf-life up to 7 days in SAGM. The shelf-life is limited by the RBCs' reaction to the harsh mechanical and osmotic conditions of freezing and thawing[38], as well as the concomitant removal of residual plasma. Together, these conditions enforce premature haemolysis and other storage lesion-related effects. Up to 20 % of the cells are lost during processing, and additionally 10 % are cleared from the circulation within 24 hours after transfusion[36]. Because of the decline in storage and transfusion quality, and the expensive and time-consuming process, cryopreservation is not feasible to the standard RBC supply. However, in situations when there is demand for a certain rare blood type, cryopreserved RBCs are obviously still highly preferable to an empty blood supply.

1.2.5.2 Irradiated red blood cells

TA-GvHD is a transfusion complication that not seldom has a fatal outcome. TA-GvHD occurs when residual donor T-lymphocytes in the transfused blood component recognise recipient antigens as foreign and activate an immune response[20]. Especially organs that are rich in HLA antigens are damaged; haematopoetic tissue, skin, liver and gut. Although leukoreduction reduces the risk of acquiring TA-GvHD with more than 90 %[39], additional safety measures are essential to ensure adequate prevention for certain patient categories.

Through irradiation (X-ray or gamma techniques[40]), the risk of inducing TA-GvHD can be further reduced[41]. During irradiation, ROS are formed, which causes DNA crosslinking to occur in the remaining leukocytes, thereby inhibiting their proliferation. At an irradiation dose of 25 Gy, the replication ability is reduced by 99.9995 % (corresponding to a reduction factor of 200 000)[42]. Therefore, the EDQM guidelines advocates that all parts of the blood component should be exposed to an irradiation dose of at least 25 Gy (maximum tolerated dose 50 Gy).

Irradiated blood components are indicated when the immune system is suppressed, impaired or not fully developed; for instance, for immuno-compromised patients (typically related to stem cell transplantation), at intrauterine transfusions, for neonates with birth weights < 1200 g, and where transfusion between relatives cannot be avoided (particularly where the donor is homozygous for HLA alleles that the recipient is heterogenous for)[1,13,43,44].

Effective irradiation of RCCs is possible because RBCs themselves do not possess a DNA-containing nucleus. However, irradiation induces premature haemolysis and an increase in ROS that damages lipids and proteins in the RBC membrane[45-48]. It also profoundly increases the leakage of K^+ to the surrounding supernatant[21,49,50]. To counteract these negative effects, described in more detail in **Chapter 1.3.2**, irradiated RCCs are typically given a shelf-life reduction. In Sweden, the shelf-life can be reduced with up to 28 days; ⅔ of the shelf-life of

conventional RCCs, since Sweden applies a "14 + 14 days policy": RCCs should not be older than 14 days when they undergo irradiation, and irradiated RCCs must be transfused within 14 days after irradiation[51]. Reducing the shelf-life is a measure that increases patient safety, but obviously, implicates an additional burden on the blood supply. Therefore, irradiation is only executed on demand, or applied to a smaller part of the RBC supply.

1.2.5.3 Washed red blood cells

RCCs can be washed in order to remove the residual plasma left after primary processing, and/or any accumulated RBC storage debris. Though the main indication for washing RCCs in Sweden is previous anaphylactic transfusion reactions, washing is also a measure to improve the safety for patients with an IgA deficiency or sensitivity to high potassium (K^+) concentrations[1,19,50,52-54]

As for cryopreserved RBCs, the ACP 215 was a milestone in the development of a closed-system, automated washing device yielding a standardised RCC[54]. However, also the mechanical stress from washing imposes severe stress onto the RBC membrane, and similar to irradiated RCCs, a "14 + 14 days" processing/shelf-life policy is applied in Sweden to ensure that washed RCCs are safe to transfuse[51]. Because of the shelf-life reduction, washing of RCCs is, similar to irradiation, only performed on demand, or applied to a selected part of the RCC supply. Recently, it was indicated that the storage lesion may be less pronounced if RCCs are washed manually instead of with ACP 215. In addition, plasma and IgA was shown to be more efficiently removed than with the ACP 215[19]. This could potentially have a positive impact on the future shelf-life of washed RCCs.

The washing principle is normally the same whether an automated or manual method is applied: the RBCs are suspended in a large volume of NaCl-based wash solution and the supernatant is washed away, during one or several cycles. In the end, the RBCs are re-suspended in new additive solution[33]. The ACP 215 was originally developed for military purposes, providing an easy-to-use, robust system applicable for standardised RCC deglycerolisation and washing by non-transfusion professionals aboard aircraft carriers[55]. The robustness of the system implicates limitations for diversity in clinical usage. One limitation is the fixed bowel volume. Independent of pre-wash RCC volume or haematocrit, output volume will be constant, meaning that for RCCs with a volume or haematocrit in the lower range of the acceptance criteria, haematocrit may not be in conformity with these criteria in the final component. Manual washing allows for a more tailored process and component outcome, but requires higher technical skills among the staff and more frequent process monitoring to limit deviations.

1.2.5.4 Pathogen reduced red blood cells

Pathogen reduction (PR) of blood components is an intervention predominantly executed for the purpose of reducing the risk of transfusion-transmitted infectious diseases and bacterial contamination. The technology behind PR, generally referred to as 'inactivation' of pathogens, is based on impairment of the replication via irreversible crosslinking of nucleic acids within

the pathogens' DNA/RNA. The treatment is a pro-active measure, inhibiting any present pathogens without necessitating a previous screening assay. Since everything in the component that has a nucleus is exposed, any present leukocytes are inhibited from proliferating by the same principle[56,57].

For plasma and platelets, where commercial systems are already available on the market, the technology is based on activation by ultraviolet (UV) light, with or without the presence of a UV photosensitiser[58]. Cerus Corporation (Concord, CA, USA) bases their inactivation technology, the Intercept Blood System, on UV-A light. Since haemoglobin light absorption maximum (400 nm) coincides with UV-A light and would render a UV-A based inactivation approach inefficient, PR of RBCs requires a different approach. Therefore, Cerus is currently developing a system for RBCs where UV light is not required. In the RBC system, the active compound is called amustaline (S-303). Amustaline is a small, positively charged alkylating agent, that intercalates with the negatively charged DNA/RNA helix. During a pH shift, triggered by the relationship between amustaline, the RCC and the integrated Intercept processing solution (adenine, mannitol, sodium citrate, mono- and disodium phosphate), amustaline is hydrolysed and the non-reactive degradation product S-300 can be removed through centrifugation. To mitigate the risk of non-specific nucleophile reactions with RBC surface antigens, the antioxidant glutathione (GSH) is added to the process as a quencher[56,57].

PR of RBCs through the Intercept method has proven efficient in the inactivation of a number of different bacteria, viruses and protozoa[59-61] as well as of leukocytes[62]. The exchange of supernatant obtained via the centrifugation step has been suggested to improve patient safety even further, through removal of the residual plasma and thus plasma proteins including IgA and HLA antibodies associated with transfusion-related acute lung injury (TRALI). Accumulated RBC debris including K^+ is washed away by the same principle [63,64]. *In vitro* and *in vivo* studies assessing the storage quality and clinical function of PR-treated RBCs show similarities to conventional, untreated RBCs[63-66], but published studies on the function as a substitute to secondary processing, paediatric use or storage in non-DEHP plasticised blood bags are not yet available. Currently, the system is still awaiting market release.

No other system for PR of RBCs has yet been presented that provides comparable RBC quality to Intercept[67-69].

1.3 RED BLOOD CELL BIOLOGY AND STORAGE LESION

1.3.1 Red blood cells

RBCs, also referred to as erythrocytes, are 7–8 µm Ø blood cells whose main task in the human body is to deliver oxygen to the tissues. They are formed as biconcave disks with a volume of about 90 fL and a large membrane area (approximately 50 % more than necessary for containing their volume) which makes them flexible and deformable enough to enter through capillaries, where the gas exchange takes place. The RBC membrane is based on an asymmetrically distributed phospholipid bilayer. The membrane skeleton comprises of more than 50 different proteins, including the blood group antigens.

The RBC originates from hematopoietic stem cells. It differentiates via numerous multipotent and erythroid-specific precursor cells, while undergoing highly specific morphological and functional changes. A key step of the erythropoiesis is when erythroblast turns into a reticulocyte by expelling its nucleus in the form of a pyrenocyte. The pyrenocyte is quickly cleared from the circulation by macrophages, while the reticulocyte matures into the red blood cell. The overall organelle clearance involves the Golgi apparatus, the endoplasmic reticulum, mitochondria and ribosomes. The lack of a nucleus and organelles, in combination with an advanced membrane remodelling process, is believed to be evolutionary advantageous to optimise the RBC haemoglobin content and cell flexibility for capillary passage [70] .

Each RBC contains approximately 270 million haemoglobin molecules. Each haemoglobin molecule consists of four globin chains, which, in turn, contains a ferrous iron (Fe^{2+})-containing haem group. The haem group has affinity for oxygen (O_2) but also carbon dioxide (CO_2) and carbon monoxide (CO) ***(Figure 3)***[71,72].

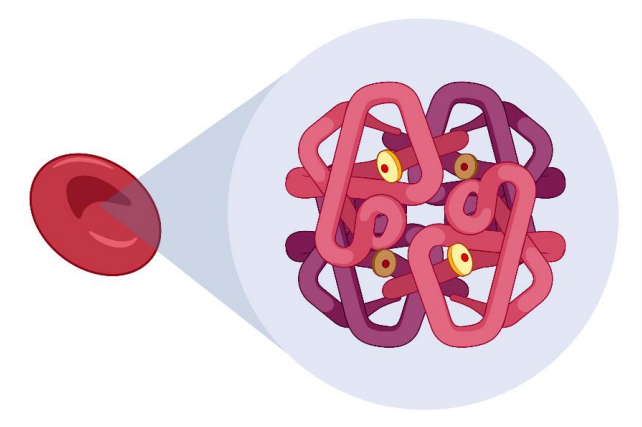


Figure 3. *Schematic overview of the red blood cell focusing on its two pairs of globin chains with one centrally placed, ferrous iron (Fe^{2+})-containing haem group per chain.*

1.3.2 Red blood cell storage lesion

The lifespan of an RBC is approximately 100–120 days in the circulation. However, when RBCs are stored at 2–6 °C blood bank conditions, they undergo a number of storage time-dependent metabolic, morphological, oxidative and enzymatic changes that dramatically influence their survival. These changes are collectively referred to as RBC storage lesion[30,31].

1.3.2.1 Red blood cells and the glycolysis

Since RBCs do not contain mitochondria, they are unable to use the citric acid cycle for ATP generation. Instead, they depend almost exclusively on anaerobic glycolysis, where glucose is metabolised into pyruvate, then lactate. In this process, the net gain for every metabolised molecule of glucose is two molecules of ATP, two reduced NADH molecules, two lactic ions and two hydrogen ions (H^+)[73] ***(Figure 4)***. The storage time-dependent accumulation of H^+ and lactate gradually increases the acidity of the RCC. When the acidity increases and pH decreases, hexokinase and phosphofructokinase-1, two key enzymes of the early stages of the

glycolysis, are inhibited ***(Figure 4)***, resulting in reduced ATP and NADH generation. NADH is a coenzyme involved in a number of metabolic reactions, including driving the conversion of pyruvate to lactate. It also has a central role in the reduction of methaemoglobin to haemoglobin, the oxygen-carrying form of the haemoglobin molecule, as presented further in **Chapter 1.3.2.4**[22].

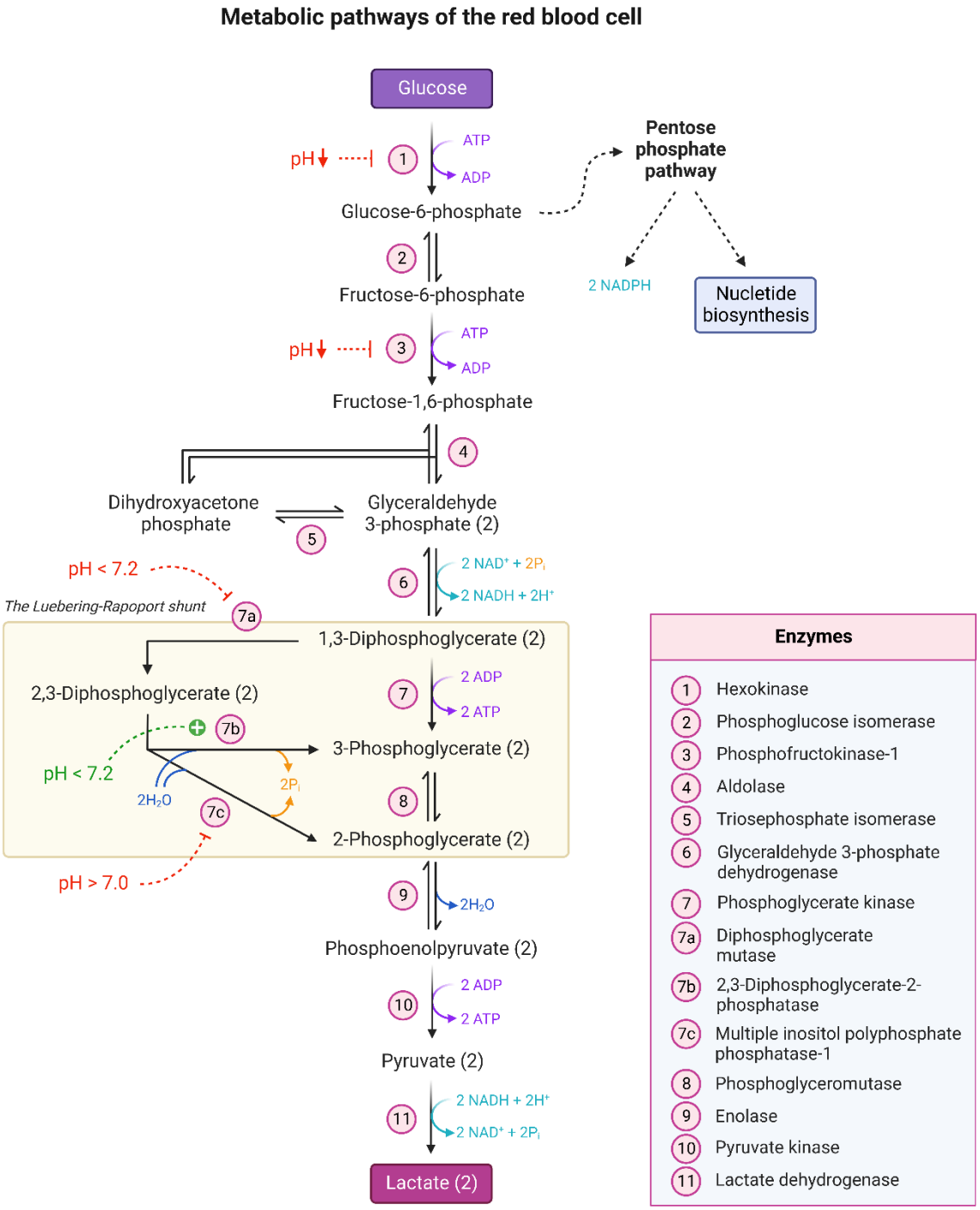


Figure 4. *The metabolic pathways of the red blood cell, including the pH-dependent Luebering–Rapoport shunt involved in the regulation of 2,3-diphosphoglycerate. The red blood cell depends completely on anaerobic metabolism. Two molecules of ATP are invested in the earlier steps of the pathway, which leads to a gain of four ATP molecules during the latter steps.*

In RBCs, > 90 % of the glucose is metabolised via the glycolysis. In addition, limited amounts of glucose are metabolised via the pentose phosphate pathway, also referred to as the hexose monophosphate shunt[74]. Metabolisation of glucose via the pentose phosphate pathway impacts the conversion of nicotinamide adenine dinucleotide phosphate ($NADP^+$) to its reduced form NADPH, another coenzyme. NADPH is essential for quenching of ROS and protection of lipid peroxidation/protein oxidisation via its role in reducing glutathione disulfide (GSSG) to GSH ***(Figure 5)***[22,73,75,76].

One of the ATP generating steps of the glycolysis is the dephosphorylation of 1,3-diphosphoglycerate to 3-phosphoglycerate via the enzyme phosphoglycerate kinase ***(Figure 4)***[73]. At a pH below 7.2, an alternative pathway is favoured: 1,3-diphosphoglycerate is rearranged into 2,3-diphosphoglycerate (2,3-DPG) by DPG mutase, then dephosphorylated into either 3-phosphoglycerate (by 2,3-DPG-2-phosphatase) or 2-phosphoglycerate (by multiple inositol polyphosphate phosphatase-1). This alternative pathway is also known as the Luebering–Rapoport shunt. An RCC initially has a pH around 6.9–7.0 at 37 °C (as demonstrated in **Papers I**–**IV**), which decreases as lactate and H^+ accumulate during storage. The Luebering–Rapoport shunt is thereby active, via an inhibition of DPG mutase and increased activity of 2,3-DPG-2-phosphatase and multiple inositol polyphosphate phosphatase-1[77,78]. This results in a depletion of the RCC 2,3-DPG levels within 14–21 days after collection[77].

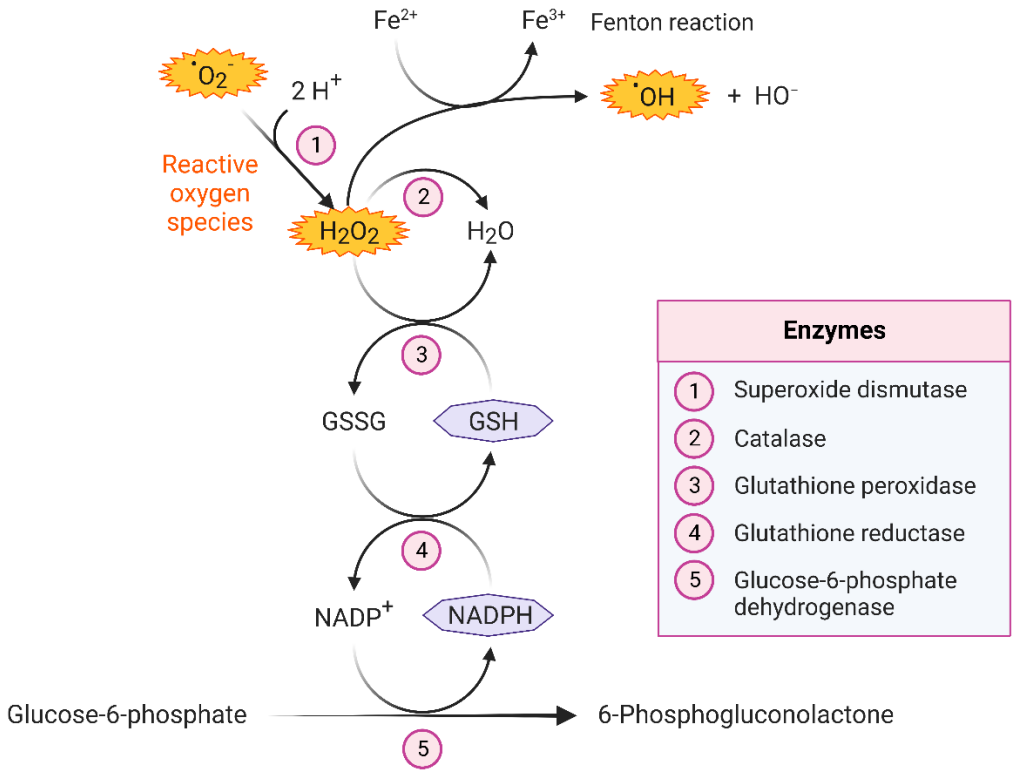


Figure 5. *Enzymatic and antioxidant (GSH and NADPH) quenching of reactive oxygen species. The conversion of glucose-6-phosphate to 6-phosphogluconolactone represents the first step of the Pentose Phosphate Pathway.*

The relationship between ATP, 2,3-DPG and pH has been demonstrated in several studies[26,27] including **Paper IV**. At decreased pH, such as the consequence of PR **(Paper IV)**, the concentration of 2,3-DPG is reduced faster than in a conventional (non-PR) RCC. This is reflected in a corresponding initial enhanced ATP generation ***(Figure 6)***. The sooner 2,3-DPG is depleted, the faster the ATP-generating dephosphorylation of 1,3-diphosphoglycerate to 3-phosphoglycerate via phosphoglycerate kinase becomes the only step in this bifurcation, thereby allowing more ATP to be generated compared to when the Luebering–Rapoport shunt is active.

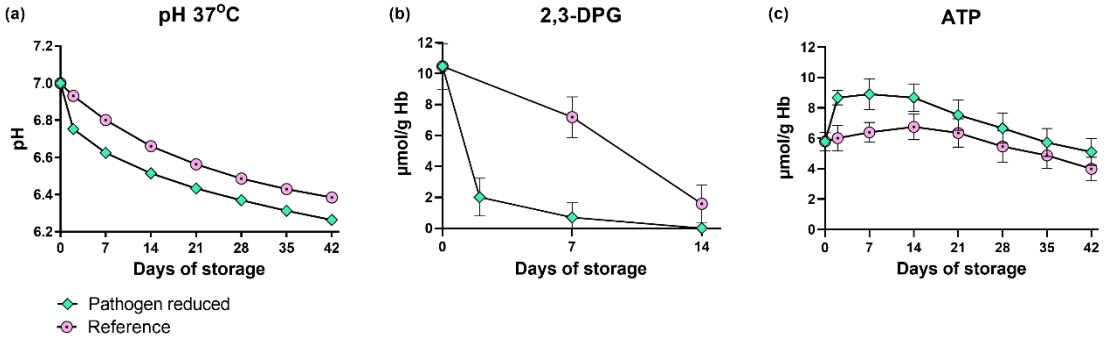


Figure 6. *Relationship between pH, 2,3-DPG and ATP during RBC storage: (a) Decreased pH from pathogen reduction induced (b) premature 2,3-DPG depletion and (c) corresponding increase in ATP; results from* ***Paper IV****. Green diamond = pathogen reduced RCCs, pink dotted circle = conventional RCCs. Data are presented as mean ± standard deviation.*

1.3.2.2 The role of ATP

As the glycolytic activity declines as a consequence of the decreasing pH, the ATP production decreases. The reduction in ATP is linked to post-transfusion *in vivo* survival of the RBCs. A remaining minimum level around 2.5 µmol/g Hb has been suggested to be necessary to ensure a 75 % 24-hour survival in the circulation[79]. In addition, a declining ATP generation rate impacts a number of functions within the RBC, including:

- The capacity of the membrane-bound Na^+/K^+ ATPase that controls the ion equilibrium over the RBC membrane is exhausted. The reduction in activity is enhanced by the low storage temperature. As the capacity is diminished, more K^+ diffuses out of the RBC cytosol than what is actively transported back into it, and elevated supernatant K^+ concentrations are seen in the RCCs as a consequence[30]. Irradiation further enhances the leakage of K^+ into the RCC supernatant by inhibiting the Na^+/K^+ ATPase[21].

- Maintaining the membrane phospholipid asymmetry. A reduction in ATP availability has been linked to a decrease of aminophospholipid translocase (flippase) activity, a membrane enzyme responsible for the active transportation of phosphatidylserine from the outer to the inner leaflet of the RBC bilayer membrane[80,81]. When ATP decreases in the RBC, there is a progressive translocation of phosphatidylserine from the inner to

the outer leaflet[82]. Phosphatidylserine is a phospholipid that functions as a signal for circulation removal through erythrophagocytosis by macrophages[83,84] and it has also been suggested to be involved in inflammation by increasing the adherence of RBCs to endothelial cells[85] ***(Figure 7).***

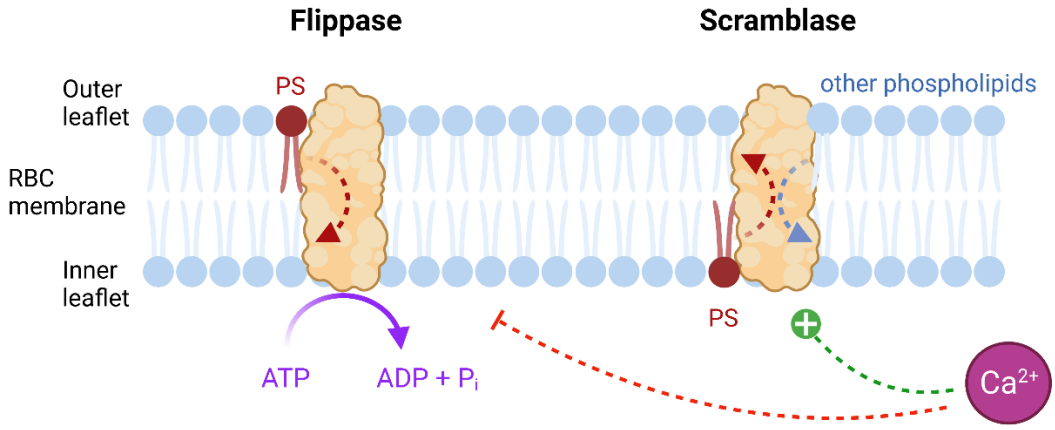


Figure 7. *Flippase moves phosphatidylserine (PS) from the outer to the inner leaflet of the RBC membrane. This action upholds an assymetry across the membrane. When the concentration of ATP declines, the flippase activity is reduced and the assymetry becomes more difficult to maintain. Scramblase transports membrane phospholipids in both directions, following the concentration gradient (counteracting flippase). Intracellular* Ca^{2+} *stimulates the scramblase activity and inhibits the flippase activity, which further decreases the phosphatidylserine asymmetry*[73,81].

- The capacity of the membrane-bound calcium ion (Ca^{2+}) ATPase involved in the regulation of Ca^{2+}. When the ATPase activity declines, the intracellular Ca^{2+} concentration increases. Ca^{2+} is involved in a number of regulatory mechanisms affecting the cytoskeletal flexibility and membrane stability, which are disrupted when the Ca^{2+} concentration increases. In addition, increased intracellular Ca^{2+} levels further stimulates the increase of K^+ in the supernatant. It is also stimulates the membrane scramblase activity and inhibits the flippase activity, which contributes further to the phosphatidylserine externalisation process[80,86,87] ***(Figure 7)***.

- Maintaining the morphological shape of the RBC. As a consequence of ATP depletion, RBCs lose membrane and undergo a shape change; turning from biconcave, discoid cells with large surface area optimised for physical capillary flexibility and gas exchange, to spiculated echinocytes, which, if the ongoing degradation process is not reversed through different rejuvenising processes, eventually irreversibly emit the top of the spiculas as RBC microvesicles (RMV)[88-90]. After RMV emission, the RBCs remain as dense, rigid spherocytes with reduced membrane area and oxygen-carrying capacity, before they eventually rupture ***(Figure 8)***. Because of the ridgidness, spherocytes may cause capillary occlusion. Occlusion in the spleen makes the RBCs extra susceptible to macrophagal removal[31].

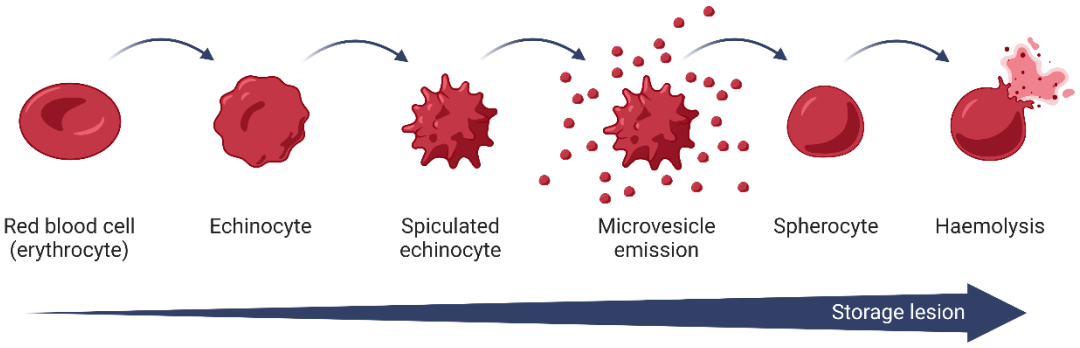


Figure 8. *The morphological shape change of an RBC as a consequence of storage lesion.*

1.3.2.3 RBC microvesicles (RMV)

RMVs are heterogenous in size (usually considered to range from 0.1 to 0.9 µm Ø) and membrane composition. RMVs contain haemoglobin from the mature RBC, and it has been suggested that more than 50 % of the "haemolysis" detected in spectrophotometrical analyses may, in fact, be RMVs[91]. RMVs are, as already described, irreversibly emitted from the spiculas of echinocytic RBCs as a response to reduced ATP levels, either because of RBC storage time, or modulated by differences in storage media[83,92-94], but they may also be a consequence of antioxidant depletion/increased presence of ROS. An example of the latter is the process of irradiation of RBCs, where hydroxyl radicals are formed. The hydroxyl radicals damages nucleotides in the leukocyte DNA, but they concomitantly induce oxidative damage of proteins and lipid peroxidation in the RBC membrane. In **Paper III**, we demonstrated an increased RMV generation rate after irradiation of RBCs, which is in line with previous research[45,46].

The lipid and protein composition of the RMV membrane seem to vary with the RBC storage environment, RMV size, isolation method and reason behind their formation[91,92]. The RMV membrane differs from the membrane of the mature RBC; an enrichment in phosphatidylserine, CD47, cholesterol, acetylcholinesterase and band 3 senescence cell antigens have been indicated in some of the RMV subpopulations[83,95]. It is believed that this accumulation is a way for the mature RBC to prolong its lifespan by escaping macrophage-instigated elimination from the circulation[75,96]. The high presence of CD47 likely constitutes of the pro-phagocytic, conformationally changed form of CD47, as presence of CD47 on young RBCs is normally considered an anti-phagocytotic structure[97]. Several studies, including **Paper IV**, have reported a percentual decrease in the number of RMVs exposing phosphatidylserine and CD47 over time, whereas the percentage of mature RBCs exposing phosphatidylserine is significantly lower but remain unchanged[83,96]. The mechanism and signification of the decrease remains to be further explored.

In the laboratory environment, measurement of RMVs can indirectly indicate the spherocytic status of the RBCs: the higher the RMV count, the more RBCs have likely turned into spherocytes.

1.3.2.4 The role of reactive oxygen species (ROS)

There are two ionised forms of iron: the ferrous ion (Fe^{2+}) and the ferric ion (Fe^{3+}). In order to carry oxygen, the iron ions of the haem molecule in haemoglobin needs to be in the Fe^{2+} conformation. The ionic charge of the iron ion changes with the loading and unloading of oxygen in the presence of water, and sometimes, Fe^{2+} is spontaneously oxidised to Fe^{3+}. This forms the methaemoglobin molecule, together with a superoxide radical ($^{\bullet}O_2^-$). In fresh RBCs, the supplies of superoxide dismutase and methaemoglobin reductase are enough to quickly correct this 'error', but when the levels of antioxidants, for instance NADH, NADPH and GSH, are reduced during storage[75], the generation of methaemoglobin and $^{\bullet}O_2^-$ radicals becomes more difficult to control**.** Methaemoglobin is an unstable molecule that quite easily loses Fe^{3+}. When free Fe^{3+} reacts with $^{\bullet}O_2^-$ and is reduced back to Fe^{2+}, at the same time as 2 $^{\bullet}O_2^-$ form the hydrogen peroxide radical (H_2O_2), the Fenton reaction is very easily instigated ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + {}^{\bullet}OH$)[98]. The Fenton reaction creates hydroxyl radicals ($^{\bullet}OH$) ***(Figure 5)***. Of the different ROS, $^{\bullet}OH$ is the most potent radical, which induces the most critical oxidative damage to the different RBC membrane structures**.**

Several types of RBC damage can be attributed to an increase in ROS and concomitant reduction in antioxidants, including membrane lipid peroxidation and protein oxidation. Aggregation and structural changes of band 3 and band 4.1 of the membrane skeleton have been demonstrated, as well as phospholipid asymmetry disruptions and inhibition of key membrane transport driving enzymes such as different ATPases[21,45,46,76,96].

In addition, Fe^{3+} is unable to bind oxygen, so an increased concentration of methaemoglobin reduces the oxygen carrying capacity. The cumulative oxidative stress impacts the integrity of the RBC membrane until the cell eventually haemolyses and thereby loses its viability. When the RBC haemolyses, free Fe^{3+} is released, which further triggers the chain of oxidative events. The oxidative damage is enhanced during conditions that increase the oxidative stress, such as irradiation[45-47].

1.3.2.5 pH and the chloride shift

The negative effects caused by the storage time-dependent decline in glycolysis rate ***(Figure 9)*** can, to some extent, be mitigated by manipulating pH. For instance, as there is a direct correlation between pH and temperature, the pH decline can be deferred by controlling the temperature[99]. In an RBC, pH is approximately 0.5 units higher at 4 °C than at 37 °C, and hence, refrigerated post-processing storage of RCCs and post-collection cooling of WB from 37 °C to RT is strongly advantageous.

Furthermore, by introducing measures that increases the intracellular pH in the RBC to ≥ 7.2, the breakdown of 2,3-DPG can be delayed[27,77,100,101]. One such strategy is the use of chloride (Cl^-)-free additive solutions: in a process that is referred to as "the chloride shift", the chloride-free additive solution forces Cl^- to diffuse out of the RBC to establish equilibrium in the surrounding supernatant. As a response, to re-establish electrical neutrality, there is a reversed influx of hydroxide ions (OH^-), the only anion that is able to diffuse into the RBC. An increased

intracellular concentration of OH^- raises the intracellular pH of the RBC. In SAGM storage, the Cl^- concentration is already higher outside the RBC than inside it. Thereby, no chloride shift takes place, and intracellular pH remains acidic, impairing the ATP generation. Although the Cl^- concentration in PAGGSM has been reduced to half compared to SAGM (72 vs. 150 mmol/L), which may contribute to the improved ATP preservation seen with PAGGSM[14,27] as demonstrated in **Papers II and III**, the pH of the PAGGSM still remains acidic (pH_{SAGM} 5.7; pH_{PAGGSM} 6.0). Experimental additive solutions, such as PAG3M, E-Sol 5 and AS-7, are all alkalic solutions completely free of Cl^-. They have a corresponding pH > 8.0 and have been linked to improved glycolytic activity, foremost via the generation of higher initial ATP and/or 2,3-DPG concentration and a slower decline in 2,3-DPG[27].

A practical issue with alkalic additive solutions is that glucose caramelises at alkalic pH during heat sterilisation[102]. This functional dilemma, together with the cost and efforts required for regulatory approval processes and component evaluations to implement a new additive solution, has likely contributed to that SAGM, to this day, remains the first-hand choice in Europe despite the ongoing exploration of more beneficial additive solution compositions. WB anticoagulant solutions remain acidic for the same reasons (pH_{CPD} 5.7), but also for the increased risk of platelet activation at a pH > 7.4[22].

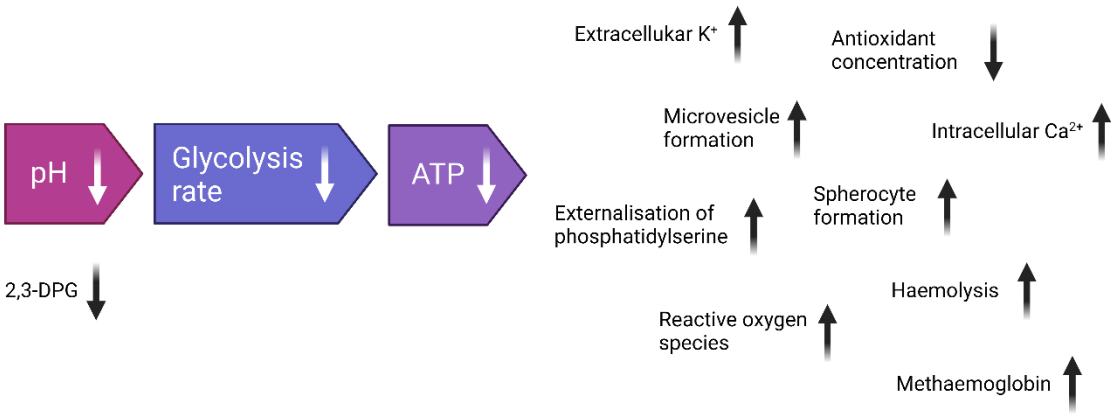


Figure 9. *When pH decreases, the glycolysis slows down, which, in turn, reduces the generation of ATP. Lower ATP concentration is detrimental to the RBC function and survival through multiple mechanisms (an exception is 2,3-DPG, which is directly affected by pH, rather than a function of ATP reduction).*

1.3.2.6 Haemolysis

Checkmate for the RBC is haemolysis; rupture of the cell membrane and release of its haemoglobin content into the surrounding media. When the RBC haemolyse, it is no longer able to carry out its oxygen delivery task. In addition, when the RBC haemolyses, haemoglobin is released from the haem pocket of the RBC and the concentration of free Fe^{3+} increases and accelerates the creation of ROS, as has been described in detail in **Chapter 1.2.3.4**.

Haemolysis is commonly calculated as % of lysed cells of the red cell mass. The maximum allowed limit in the Council of Europe member states is set to 0.8 %, independent of how long an RCC has been stored or what kind of processing interventions that has been applied to it[13]. Corresponding limit in the US is 1.0 %[103].

Haemolysis is irreversible. RBCs haemolyse when the cell degradation process has progressed too far and the membrane integrity is lost, as a result of storage lesion ***(Figure 8)***. However, haemolysis can also be induced from exposure to a number of different stressors. Cornerstones in the general processing and handling strategies for RBCs is therefore to mitigate the generation of haemolysis by limiting the cumulative exposure to conditions that induces stress.

Haemolysis from secondary processing

Some stressors are necessary secondary processing steps, like cryopreservation, irradiation or washing. The benefits of these interventions for specific patient categories, as well as the impact on shelf-life, have been described in **Chapters 1.2.5.1**–**1.2.5.3**.

- **Cryopreservation**
  Cryopreservation of RBCs is an intervention that puts an even higher total stress load onto the RBC; the cells are exposed to severe osmotic stress during both glycerol addition and removal, as well as mechanical stress during the post-thawing washing step with ACP 215[34,38]. In addition, liquid glycerol is a cytotoxic compound in itself which contributes to haemolysis during the glycerolisation/ deglycerolisation process[38]. Measures to reduce the total amount of stress during the cryopreservation process, such as omitting a centrifugation cycle, have been shown to reduce the haemolysis rate during post-thawing storage[104].

- **Irradiation**
  Irradiation creates ROS that damages the RBC membrane, both directly, through the induction of oxidation of proteins and peroxidation of lipids of the RBC membrane, as described in **Chapter 1.2.3.4**, and indirectly, via an increased release of haemoglobin and free Fe^{3+} through the induction of premature haemolysis, a process which, in turn, instigates even more ROS[45,46]. When comparing irradiated and non-irradiated RCCs, the haemolysis increases faster after irradiation ***(Figure 10)***. In addition, the older an RCC is when irradiated, the faster the haemolysis rate[49,105].

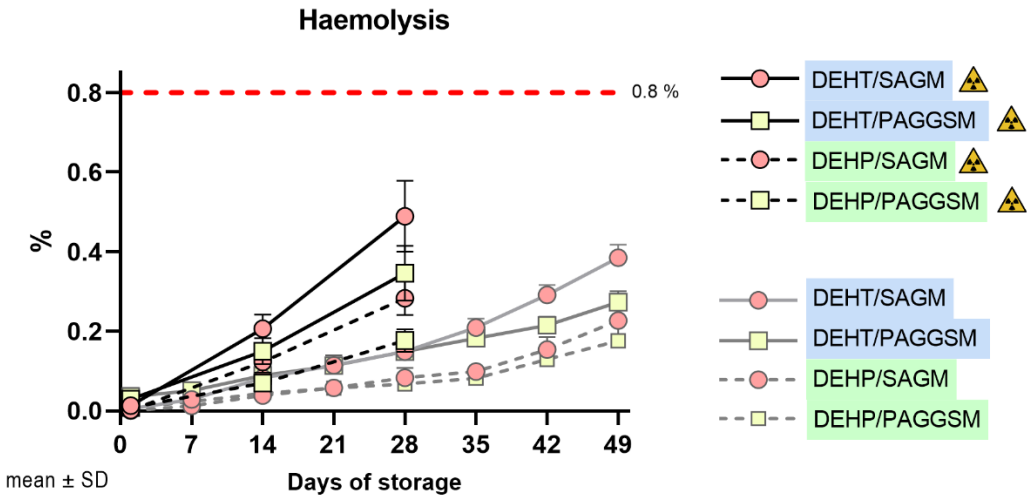


Figure 10. *Irradiation of RBCs implicates premature haemolysis. Black lines = irradiated RBCs; grey lines = non-irradiated RBCs; full lines = DEHT; dashed lines = DEHP; red dots = SAGM, yellow squares = PAGGSM. Data are presented as mean ± standard deviation.*

- **Washing**

  For washing, the generation of haemolysis is related to a high mechanical strain on the RBC membrane during the repeated centrifugation cycles involved in the process, and possibly also osmotic stress from exposure to the washing solution[54,96]. Recently, a washing protocol was proposed where manual washing using one or two centrifugation cycles proved to generate less haemolysis compared to automated washing with the commonly used ACP 215 (Haemonetics corp.). The same study proposed washing directly in SAGM, as compared to an isotonic, NaCl-based washing solution, the current common practice, as a further improvement strategy for reducing the post-washing haemolysis rate[19].

Strategies to keep the haemolysis at a minimum after secondary processing include the use of fresh RCCs, shelf-life reduction of the final RBC components, an on-demand based approach of intervention (avoiding large stock-keeping of irradiated or washed RCCs), transfusion as soon as possible post-intervention, and policies on only issuing secondary processed components where medically warranted.

Haemolysis from mishandling during processing and storage

Several storage and handling strategies can induce premature haemolysis:

- **Temperature**

  Optimal storage temperature for an RCC is "just above freezing but *not* freezing", and therefore, red cells are usually stored in 2–6 °C refrigerated compartments. If red cells are frozen during an uncontrolled process or without a cryoprotectant, ice crystals form inside the cell that causes it to swell and rupture[37]. Similarly, storage ≥40 °C impairs RBC functions and above 45 °C, severe haemolyse have been demonstrated[106]. Also in

the interval between these two extreme values, there is an increase in RBC metabolism with increased temperature, which amplifies the haemolysis rate. Because of the relation to temperature, haemolysis can be mitigated to some extent by using a collection-day separation strategy instead of storing the WB overnight in RT before processing[14,107].

- **Mechanical stress and shear stress**
  Haemolysis can be induced by too forceful or improper handling. Examples of when this can happen include:
  - During passages through narrow openings: kinked blood bag tubes, sampling needles and leukoreduction filters if filtration is not performed through gravity
  - Via sharp edges and turbulent flow from improperly broken breakaway cannulas
  - Vigorous shaking/mixing during processing or sampling, or just after an intervention that implicated mechanical stress
  - Extensive or too forceful centrifugation
  - Tube stripping during sampling

  The RBCs are even more sensitive to mechanical stress if the haematocrit is high, and a storage haematocrit ≥ 70 % is normally discouraged. This is one of the reasons behind dilution of RBCs in additive solution during storage. The RBC fragility increases in cold temperatures. Therefore, it is recommendable to let an RCC adapt to RT before exposing it to severe mechanical stress, for instance a washing procedure. Centrifugation in cold temperatures also increases the risk of enforcing premature haemolysis.

- **Osmotic stress**
  RBCs can haemolyse during conditions of hyper- or hypotonic storage, especially if the conditions change fast. Part of the haemolytic loss of RBCs during the glycerolisation/deglycerolisation procedure can be attributed to osmotic stress[34,38,96,108,109].

- **Presence of leukocytes, platelets and bacteria**
  Leucocytes release numerous haemolysis-inducing substances when they lyse, including hydrogen peroxide and different pro-haemolytic enzymes. A clear relationship between pre-storage leukoreduction and reduced haemolysis rate has been established[17,18]. Additionally, activated platelets, activation of the complement system and the presence of bacteria have all been linked to increased haemolysis levels[110,111]. Processing of the WB using the buffy coat method and subsequent leukoreduction of the RCC is an efficient way of minimising the number of platelets in the RCC. Bacterial contamination is foremost a risk if hygiene routines for donation or processing have not been properly followed.

- **Blood bag material and additive solution**
  The generation of haemolysis is strongly affected by choice of blood bag material; more specifically by the presence of DEHP, as is further described in **Chapter 1.4** and demonstrated in **Papers II and III**. In addition, surface tension between the RBCs and the blood bag creates electrostatic forces that may be an instigator of haemolysis. Therefore, there is a relationship between blood bag surface area and the volume of RBCs stored in the blood bag; i.e. the ratio between the RCC volume and the bag surface area should be kept as small as possible[112]. In addition to facilitating the involvement of fewer donors and reduce wastage, this is one of the key rationales for using paediatric-sized blood bags for storage of split RCCs[113]. As has already been discussed in **Chapters 1.2.4 and 1.3.2.5**, as well as demonstrated in **Papers II and III,** the additive solution composition also has a major impact on the metabolism rate, ATP generation, and thereby ultimately the haemolysis rate[14,25-27,77,101].

- **The individual blood donor**
  Individual donor characteristics is yet another element that impacts the haemolys generation. A factor that have been particularly highlighted is the biological sex; higher haemolysis levels seems to correlate with RCCs collected from biologically male blood donors. It has been suggested that RBCs from biologically male donors are more fragile and less resistant to mechanical and oxidative stress[105] than corresponding RBCs from biologically female donors. Relating to the previous paragraphs about haemolysis, biologically male donors also have an overall higher haemoglobin concentration than biologically female donors, which yields a higher mean haemotocrit in the RCCs when the collection volume is similar. This could also have an impact on the sex-related differences in haemolysis.

1.3.3 Clinical implications of RBC storage lesion

The expectation of transfusion of RBCs is to raise the recipient's haemoglobin concentration. In an average-sized adult (70–75 kg), administration of one RCC is approximated to increase the concentration by around 10 g/L, corresponding to an increase of the haematocrit by roughly 3 %[1]. As mentioned in **1.3.2.2**, ≥ 75 % 24-hour circulatory post-transfusion survival is a long-standing quality criteria for transfusion of RBC components[13,79]. Heavily progressed RBC storage lesion does not only challenge these expectation in terms of the count of viable RBCs: In addition, there are two main considerations for the clinical implications of the storage lesion: the RBC *in vivo* functionality and potentially toxic effects for the recipient.

A haemolysed RBC is unable to carry oxygen or carbon dioxide, and it thereby loses its principal function. Consequently, it will not help in correcting an anemic condition. RBCs that exhibit pro-phagocytotic surface markers, such as phosphatidyl serine, are quickly cleared from the circulation by macrophages, and in other words, also not of much help to the patient[82-84]. Thus, the longer the storage time, and the more advanced the storage lesion, the less the actual effect is of a transfusion. The oxygen-carrying effect is also impeded when the RBCs lose

membrane through microvesiculasation and becomes spherocytes. In addition, because of their reduced flexibility, spherocytes can cause capillary occlusion.

2,3-DPG facilitates oxygen unloading from the RBC. Depletion of 2,3-DPG is associated with a higher oxygen affinity and thereby reduced oxygen delivering capacity. 2,3-DPG is normally restored within a few days after transfusion[114,115] and thus, depletion of 2,3-DPG in an RCC for a top-up transfusion is not overly alarming. However, to paediatric patients or at massive transfusion, where an efficient oxygen delivery is more critical, 2,3-DPG depletion may still be a critical defect.

Large amounts of free K^+ can induce transfusion-associated hyperkalaemia, which has been linked to cardiac arrhythmia and arrest in a number of case studies[116-118].

Free Fe^{3+} from haemolysis stimulate the generation of ROS that damage both other RBCs and the surrounding tissue. They also decrease the availability of nitric oxide (NO) in the vascular system. NO plays in important role as regulator of vascular dilatation and is a potent inhibitor of platelet and monocyte adhesion, platelet aggregation and the expression of several proatherothrombotic molecules including tissue factor. Thereby, a consequence of increased ROS and haemolysis is vasoconstriction, inflammation and platelet activation[119,120]. Too high levels of Fe^{3+} can also be toxic to multiple body functions and can cause coagulopathy, metabolic acidosis and permanent organ damage[121]. In addition, Fe^{3+} stimulate bacterial growth[122].

The clinical impact of RMVs lies in their negatively charged lipids providing pro-inflammatory and pro-coagulant surfaces. Like iron ions, RMVs have been linked to ROS, inflammation and thrombosis[123,124].

1.4 PLASTICIZERS IN BLOOD BAGS

1.4.1 Di(2-ethylhexyl) phthalate (DEHP) – what's the deal?

A PVC blood bag requires a plasticizer in order to be soft, flexible and withstand processing and everyday handling without risking breakage and component loss[2]. The plasticizer DEHP provides these benefits. Belonging to the group of chemical substances called phthalates, DEHP is an esterification of the anhydride of phthalic acid and two 2-ethylhexanol molecules in ortho configuration ***(Figure 11)***[125].

DEHP is favourable for storage of RBCs. In the blood bag plastics, DEHP is bound non-covalently to the PVC matrix. Being a lipophilic, dipolar molecule, it leaches into the stored blood component and incorporates into the RBC membrane, where it has a stabilising effect. Consequently, DEHP aids in preserving the RBC membrane integrity. This effect considerably mitigates haemolysis, which largely contributes to making the current practice of 35–42 days of RCC shelf-life possible. DEHP is also linked to reduced RMV formation and increased RBC post-transfusion survival in the circulation[126-128]. Several studies, including one performed at the blood establishment of Karolinska University Hospital, have linked non-DEHP blood bags to unacceptable haemolysis levels[113,129,130]. Because of its membrane-protecting attribute, DEHP has remained the almost exclusive plasticizer of choice for RBC storage for more than sixty years.

The use of DEHP is, however, a two-sided coin, as endocrine-disruptive toxicity has been reported in rodents[3,131]. The toxicity is linked to the primary metabolite of DEHP, mono-2-ethylhexyl phthalate (MEHP) [132]. Though actual evidence of toxic impact on the human species is lacking, it cannot be completely excluded that DEHP and/or its metabolites may be harmful at extensive exposure such as repeated transfusions or frequent apheresis donations.

These concerns have encouraged a revision of the EU legislation concerning the use of phthalate plasticizers in medical devices including blood bags, where the Regulation (EU) 2017/745 on Medical Devices (MDR) is the key regulatory document restricting the usage[133]. This regulation states that maximum content of 0.1 % weight/weight of DEHP will be allowed unless a benefit-risk assessment can prove that the absence of DEHP is more hazardous than the use[134]; something that could possibly be argued for RBC storage, but not for the other components derived from the same blood bag system. The ban is encouraged by the European Chemical Agency (ECHA) that recommends the European Commission to amend the Authorisation List (Annex XIV of REACH, "Registration, Evaluation, Authorisation and Restriction of Chemicals") with DEHP[135]. In practice, this means that the previous exemption of using DEHP in blood bags ceases to exist, with an overall sunset date for the commercialisation of DEHP-containing blood bags by May 27th, 2025.

As an issue on its own, it has been proposed that blood bag systems should be upclassified from MDR class IIb to class III, which may impose a need for clinical studies to forego market authorisation of any new blood bag systems. At present time, a final decision has not been

reached, but if such an upclassification is implemented, the complexity of the non-DEHP transition will increase further.

1.4.2 Non-DEHP plasticizer alternatives

In light of these regulations, finding a substitute plasticizer with a non-toxic profile is a very high priority, but an important requirement is that such a substitute should not compromise the RBC quality[4]. 1,2-Cyclohexanedicarboxylic acid, diisononyl ester (DINCH) and di(2-ethylhexyl) terephthalate (DEHT) are two plasticizers that has shown quite tolerable RBC quality in storage studies, albeit still not equivalent to that of DEHP[29,113,130,136,137]. DINCH in particular has been investigated as a substitute for some time, and there are already commercially available dry storage bags, designed for paediatric applications[113]. To the best of the author's knowledge, only a single study has been published about DEHT in a blood bag concept prior to **Papers II and III** included in this thesis[136]. In addition, n-butyryl tri-n-hexyl citrate (BTHC), a plasticizer primarily used for platelet storage bags, has recently gained new interest to be explored as potential DEHP substitute also for RBCs[138], after some previous demonstrations of inferior storage properties compared to DINCH and DEHT[113,130].

The removal of DEHP is foremost an RBC concern, as no differences in plasma or platelet quality or function have been demonstrated during DEHP-free storage[29,139]. **Paper III** supports these conclusions.

However, it is not only the RBC quality that needs to be considered. Except for storage lesion, it is imperative that a substitute plasticizer also possesses physical properties appropriate for all the different processing and handling procedures of modern blood banking. These characteristics include, among others, withstanding sterile welding, sealing, quick changes in temperature (cooling, freezing, thawing and warming), repeated centrifugation and transport in boxes and pneumatic tube systems without breaking or leaking, as well as ergonomic and work environment-related factors for collection and processing operators. Of course, it is also of utmost importance that an alternative plasticiser does not introduce another kind of toxicity.

1.4.3 Di(2-ethylhexyl) terephthalate (DEHT)

DEHT is a structural isomer of DEHP, but the two 2-ethylhexyl esters are in para position to one another ***(Figure 11)***. This makes DEHT a terephthalate that is completely hydrolysed to terephthalic acid without monoester formation[140]. This difference in ester configuration appears to be central for the toxicity pattern of DEHT, as no similar effects have been linked to this plasticizer as those related to DEHP[141-143]. Additionally, leaching rate into the blood component seems to be markedly lower compared to DEHP[136].

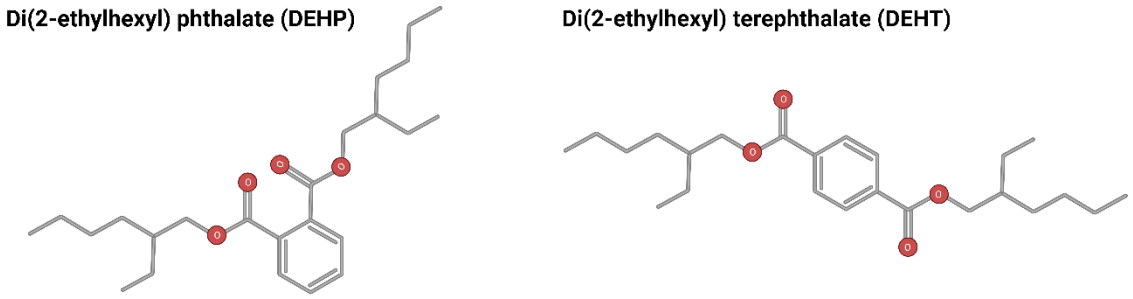


Figure 11. *Comparison of the structural formulas of DEHP and DEHT. In DEHP, the two 2-ethylhexyl esters are in ortho configuration whereas in DEHT, the similar structures are in para configuration.*

Storage of RBCs in PVC-DEHT was recently investigated with PAGGSM and AS-1 (saline-adenine-glucose-mannitol; the US equivalent to SAGM) as additive solutions[136]. The study demonstrated encouraging day 42 haemolysis levels in DEHT storage (0.38 ± 0.10 % with PAGGSM, 0.49 ± 0.13 % with AS-1), even though it was higher than in the DEHP reference (0.32 ± 0.07 %). In particular, it was proven that PAGGSM could partially mitigate the haemolysis generation. Neither of the metabolic parameters were affected by non-DEHP storage, which supports previous studies of other plasticizers indicating that the removal of DEHP primarily affects the RBC membrane stability as opposed to the metabolism[29]. A shortcoming of this study was that the WB was transferred to PVC-DEHT storage bags only after initial collection in PVC-DEHP; leaving the impact on the RBC membrane from early DEHP contamination undetermined. Prior to the studies conducted for **Papers II and III** of this thesis, a knowledge gap remained on the RBC storage lesion from storage in DEHT/SAGM, as well as on storage in DEHT as exclusive plasticizer.

2 RESEARCH AIMS

The overall research aim of this PhD project was to develop new or more efficient strategies to process and store both new and established RBC components. The purpose was to reflect the predicted future demands in terms of quality and blood supply awareness while accommodating to new regulatory frameworks.

A subsequent aim was to update and improve the battery of storage lesion analyses at the Karolinska blood establishment by introducing flow cytometry as a technique to study microvesicle emission from the RBC membrane in terms of both count and composition, and also re-assess the technique for haemolysis measurements by optimising sampling technique and pre-analysis handling.

Paper I

The aim of the study presented in **Paper I** was to develop a method to cryopreserve RBCs in smaller transfusion units using the automated device ACP 215. Dividing conventional RCCs into three partial units could help increase the supply of RCCs of rare blood types by reducing waste, and also facilitate the involvement of fewer donors in repeated paediatric transfusions of rare blood.

Paper II

In the **Paper II** study, we aimed to assess the quality of RBCs stored in a novel PVC-DEHT blood bag system completely free of DEHP contamination, to determine whether DEHT, paired with SAGM or PAGGSM additive solution, is a viable plasticizer substitute to DEHP in future RBC storage.

Paper III

The study presented in **Paper III** was a follow-up study to **Paper II**. In **Paper II**, we assessed storage of RBCs with the plasticizer DEHT during ideal storage conditions, but it is imperative that a new plasticizer can maintain RBC quality also when exposed to secondary processing-related stress. Therefore, we aimed to compare RBC quality, stored in a similar setting as in **Paper II**, after intervention by X-ray irradiation.

Paper IV

The study of **Paper IV** had two aims. The first was to investigate whether PR-RCCs could be stored for 42 days (current RCC shelf-life in Sweden) with acceptable *in vitro* quality. The second was to assess whether PR-RCCs can be considered as substitute to both irradiated and washed RCCs without necessitating a reduction of the proposed 42-days shelf-life.

3 MATERIALS AND METHODS

This chapter provides an overview of the materials and methods used for the conducted studies. Full details are provided within each individual paper **(Papers I–IV)**.

3.1 WHOLE BLOOD SEPARATION AND PROCESSING

WB was processed according to the standard operating procedures of the blood establishment of Karolinska University Hospital in all four studies. In short, these consist of the following main principles:

- Collection of 450 mL ± 10 % (405–495 mL) WB into quintuple bottom-and-top blood bag systems (the NPT reference, Macopharma, Mouvaux, France), containing 63 mL CPD in the collection bag
- Placement of the WB on cooling plates pre-filled with butane-1,4-diol to facilitate cooling of the WB to RT (20 °C)
- Transport of the WB from the collection sites to the processing site at Karolinska University Hospital, Huddinge
- Collection day processing: centrifugation ≥ 2 hours post-collection (3130 × *g*, 11 minutes, MacoSpin, Macopharma) and subsequent immediate extraction (MacoPress Smart Revo, Macopharma) into RCCs, plasma and buffy coat
- Addition of 100 mL additive solution to the packed RBCs during ongoing separation
- Leukoreduction of the RCCs via leukofilters integrated in the blood bag system
- Tube sealing with sealers from the Qseal range (Conroy Medical AB, Upplands Väsby, Sweden)
- Storage at 2–6 °C, all within eight hours of collection

Paper I

A pool of eight RCCs of similar ABO blood type was split into eight new RCC units (A–H) of equal content and volume. After overnight storage at 2–6 °C, they were glycerolised using ACP 215 and the associated glycerolisation set 225 (both Haemonetics Corp., Braintree, MA, USA) following the Karolinska blood establishment routine SOP (high-glycerol method, based on the Meryman method[33,35] using ethylene-vinyl acetate (EVA) bags with nominal volume 1920 ml (GSR8000AU, MacoPharma). Four glycerolised RCCs, A–D, were kept as non-split reference. The other four RCCs, E–H, were further split in three (n = 12 in total). All glycerolised RCCs were stored in similar EVA bags at ≤ −65 °C for at least 30 days.

After storage, all glycerolised RCCs were thawed to 32 ± 2 °C (SW22 water bath, Julabo, Seelbach, Germany), then deglycerolised and washed using ACP 215 (individual weights, all other settings as default; bowl volume 275 mL) following the manufacturer's instructions.

After deglycerolisation/washing and one hour rest in RT, the splits were centrifuged (1255 × *g*, 10 minutes, Macospin) to increase their haematocrit: all supernatant was removed and new

SAGM (VSE4080XU, MacoPharma) was added to a final haematocrit of 60 %. Afterwards, the split RCCs were transferred to 150 mL paediatric storage bags (VQL001XC, MacoPharma) to reduce the bag surface area exposure.

The RCCs were sampled on day 1, 2, 3, 4, 7 and 10 post-cryopreservation. To reduce the influence of volume reduction on haemolysis, split 1 and 2 were analysed on days 1–4, whereas split 3 was analysed on days 7 and 10.

Paper II

Prototype blood bag systems made of PVC were plasticized with either DEHT or DEHP. The prototypes resembled the NPT system (Macopharma internal nomenclature), divided into two parts: part I: the collection bag separately, and part II: the rest of the processing system, starting with a tube leading into to an empty collection bag ***(Figure 12)***. This separation enabled a pool-and-split strategy for optimal additive solution comparison in relation to each plasticizer. A total of 64 WB units were collected, n = 32 (blood type A = 20, O = 12) into each plasticizer. The WB units were pooled pairwise following blood type, using pooling bags of the same assigned plasticizer. Two different RBC additive solutions were used: SAGM and PAGGSM. The collection bag and the rest of the processing system were sterile connected (TSCDII; TerumoBCT, Lakewood, CO, USA) to create four different RBC plasticizer/ additive solution combinations, i.e. four different study arms: DEHT/SAGM, DEHT/PAGGSM, DEHP/SAGM and DEHP/PAGGSM. The RCCs were sampled and analysed weekly for 49 days.

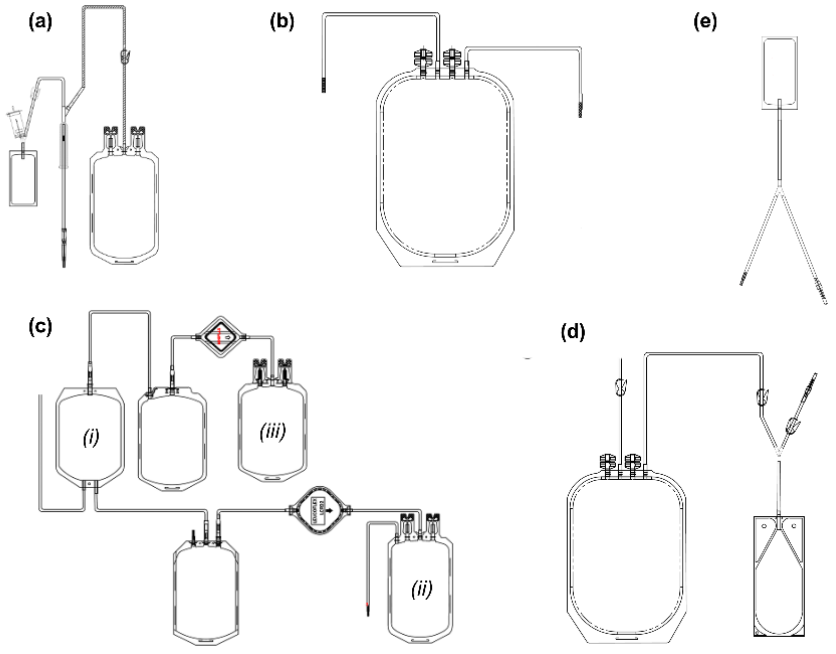


Figure 12. *Blood bag prototypes plasticized with DEHT or DEHP: (a) donation bag containing 63 mL CPD, (b) pooling bag, (c) processing system with (i) empty primary bag, (ii) red blood cell storage bag containing 100 mL additive solution, (iii) plasma storage bag, transfer bags (unmarked) and leukocyte reduction filters, (d) platelet storage bag, (e) sampling bag. Figure from* ***Supporting information 1, Paper II.***

Paper III

Similar prototype blood bag systems as in study 2 were used for this study; however, the collection bag and the corresponding processing bags were sterile connected without no prior pool-and-split procedure. A total of 59 RCCs were used for study 3, divided into four study arms (DEHP/SAGM n = 14, DEHT/SAGM, DEHT/PAGGSM and DEHP/PAGGSM n = 15; all blood type A).

All RCCs were X-ray irradiated on day 2 post-collection (25–50 Gy; Raycell Mk2, Best Theratronics, Ottawa, Canada). Sampling and analysis were performed on day 2 (pre-irradiation), day 14 (post-irradiation) and day 28 (post-irradiation) after collection.

Paper IV

RCCs were pooled four-and-four directly after processing and split into four study arms (n = 48 in total, n =12 per arm): PR, irradiation, automated washing and reference (untreated RCCs).

PR was performed on day 1–2 according to the manufacturer's specifications (Cerus Corp.). In short, the RCC was transferred to a processing set containing processing solution: adenine, mannitol, sodium citrate, mono- and disodium phosphate, and GSH and amustaline was added. The mixture was held at 20–25 °C overnight (18–24 hours). After incubation, the supernatant, containing amustaline metabolites, was removed via centrifugation (4194 × *g*, 8 minutes, 22 °C; Macospin). Finally, the RBCs were resuspended in new SAGM. The entire process was executed within a 26-hour timeframe.

Irradiation was performed with X-ray technique (25–50 Gy; Raycell Mk2, Best Theratronics, Ottawa, Canada) on day 14 post-collection. Automated washing was executed using ACP 215 (Haemonetics Corp.; bowl volume 275 mL) on day 14 post-collection. The reference RCCs were not processed further.

The RCCs were sampled and analysed on the collection day (day 0), on day 2 (post-PR processing), then weekly from day 7 until day 42 post-collection. On day 14, the irradiated and washed study arm were analysed on two occasions; before and after intervention.

3.2 SAMPLING

In **Chapter 1.3.2.6**, the impact of different stressors on the generation of haemolysis has been described. It is essential that, when sampling the RCCs for analysis, the RBCs in the sample pouch are representative of the actual status of the RCC at the given point of time, and not influenced by outer stressors.

When evaluating the Karolinska blood establishment routine sampling procedure prior to the studies included in this thesis, a number of suboptimal details were identified and improved:

- 40 mL sampling bags (VSE0000Y, Macopharma) were introduced instead of previous bags of 150 mL nominal volume, to reduce the ratio between the RCC volume and the bag surface area from 1:15 to 1:4, calculated on 10 mL sample (the bag volume is indicative of the surface area, although not identical).

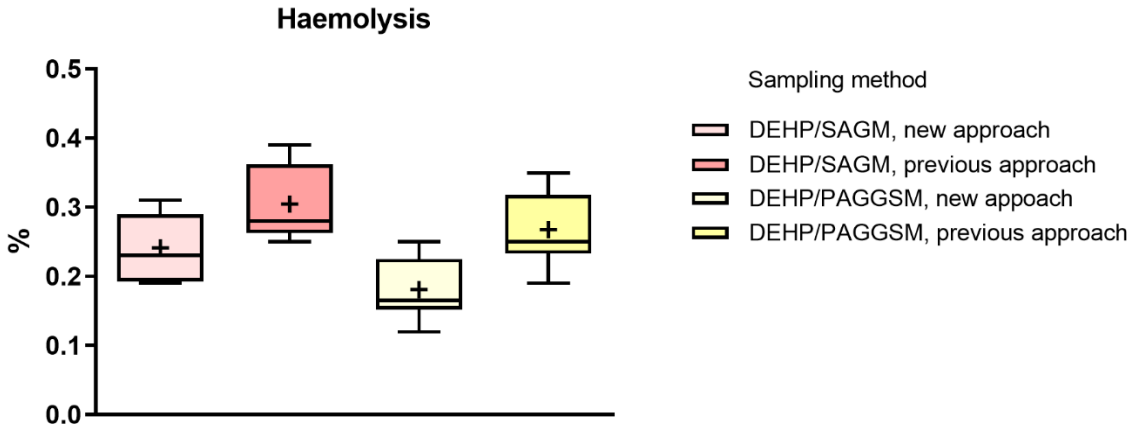
- For the same reason, and thereby minimise the impact on the remaining RCC, a more stringent control of the sample volume was introduced.

- Even in the smaller sampling pouch, the ratio between RCC volume and bag nominal volume was larger than in the corresponding full RCC; a 1:4 ratio (10 mL RCC in a 40 mL sampling pouch) compared to a 1:2.2 ratio (270 mL RCC in a 600 mL conventional storage bag). Therefore, to minimise the time of storing the RBCs in the sampling bag, a mandatory immediate start of the analysis work after sampling was introduced.

- Previously, the sampled RCC was allowed to rest on the lab bench for a considerable amount of time before start of the analysis. Therefore, it was mandatory to mix the the sampling pouch for at least 5 minutes on a mechanical mixer before the sample was further analysed, to ensure a homogen constitution of the sample in the different tubes. Because of the introduction of no delay between sampling and analysis, the RCC was freshly mixed from when the sample was drawn, and the second mixing step on the mechanical mixer could be omitted.

- In the previous routine, the analysis tubes were filled by sample from the sampling bag via a syringe. To eliminate the risk of introducing haemolysis by drawing the RBCs through a narrow passage by force, the procedure was changed and instead, the PVC tube of the sampling bag was cut open to let the RCC flow into the testing tubes by gravity.

The changes were evaluated in a pilot study (unpublished), where the combined influence of sampling pouch size and a one-hour RT rest + 5 minutes mixing was compared in both DEHP/SAGM and DEHP/PAGGSM blood bags at day 49 post-collection. The haemolysis was assessed as described in **Chapter 3.3.1.** The study clearly demonstrated that significantly higher haemolysis ($p < 0.001$) was created with the previous method independent of additive solution composition ***(Figure 13)***.



Blood bag	Sampling pouch	Mixing?	Mean	Standard deviation	*p*-value	Significant difference?
DEHP/SAGM	40 mL	No	0.24	± 0.05	< 0.001	***
DEHP/SAGM	150 mL	Yes	0.31	± 0.05	< 0.001	***
DEHP/PAGGSM	40 mL	No	0.18	± 0.04	< 0.001	§§§
DEHP/PAGGSM	150 mL	Yes	0.27	± 0.05	< 0.001	§§§

Figure 13*. Sampling pouch size and pre-analysis mixing had a significant impact ($p < 0.001$; *** = between new and previous method in SAGM, §§§ = between new and previous method in PAGGSM) on the haemolysis result. The data are presented as boxplot graphs with min–max whiskers and + marking the mean. Significance between the previous and new method were tested using paired t-test; n = 8 of each category (n = 32 in total).*

3.3 LABORATORY ANALYSES

3.3.1 RBC storage lesion analyses

Haematocrit (Hct; %), haemoglobin (Hb_{RCC}; g/unit and g/L) and mean corpuscular volume (MCV; fL)

A 3.5 mL Ellerman tube was filled by gravity with at least 2.0 mL RCC sample. The tube was vortexed, then run on Swelab Alfa Plus Basic hematology analyzer (Boule Diagnostics AB, Spånga, Sweden).

Extracellular potassium ions (K^+, mmol/L), glucose (mmol/L), lactate (mmol/L) and pH

A 2.0 mL plastic syringe was filled by gravity with at least 0.5 mL RCC sample. The syringe was run immediately on ABL 800 Flex blood gas analyzer (Radiometer Medical ApS). Where indicated, the concentrations were re-calculated into mmol/mg Hb to compensate for differences in Hct: mmol/mg Hb = (mmol/L × (1 − Hct)) / Hb_{RCC} (g/L) × 1000.

Haemolysis (%)

A 3.5 mL Ellerman tube was filled by gravity with at least 2.5 mL RCC sample. The tube was centrifuged at 1450 × *g*, 10 minutes, RT. The supernatant was pipetted into a new Ellerman tube and the centrifugation cycle was repeated. One drop of the supernatant was pipetted onto a plastic film. A Hemocue cuvette was filled and analysed using the HemoCue plasma/low haemoglobin photometer (Radiometer Medical ApS, Brønshøj, Denmark). Haemolysis was calculated as: haemolysis (%) = (100 − Hct) × $Hb_{supernatant}$ (g/L) / Hb_{RCC} (g/L).

RBC microvesicles (RMV): count (per µL supernatant) and surface markers phosphatidylserine (%) and CD 47 (%)

A 3.5 mL Ellerman tube was filled by gravity with at least 3.0 mL RCC sample. The tube was centrifuged at 2 × (2500 × *g*, 15 minutes, 4 °C, **Papers I**–**III**) or 1 × (2000 × *g*, 10 minutes, 4 °C, **Paper IV**). Immediately after centrifugation, ≥ 400 µL supernatant was pipetted into each of two Eppendorf tubes (duplicates) and stored at ≤ −70 °C until flow cytometry analysis.

The samples were thawed on 37 °C heating block until liquid state. To each TruCount tube, the following were pipetted:

- 150 µL sample **(Papers I**–**III)** or 50 µL sample **(Paper IV)** by reverse technique
- 5 µL BV421 **(Paper I)** or PE-Cy7 **(Papers II**–**IV)** mouse anti-human CD235a
- 5 µl BV421 mouse anti-human CD47 **(Paper IV only)**
- 3 µL Alexa Fluor 647 mouse anti-human Annexin V
- 100 µL annexin binding buffer
  (Annexin V: Bio-Legend, San Diego, CA; all others: BD Biosciences, San Jose, CA, USA)

After incubation for 20 minutes in the dark, RT, 300 µL additional buffer was added for volume. The samples were run immediately after incubation on BD FACS Canto II (BD Biosciences; **Paper I**) or CytoFLEX (Beckman Coulter, Brea, CA, USA; **Papers II**–**IV**). Using sizing beads (Megamix; BioCytex, Marseille, France), the microvesicle population was gated as ≤ 0.9 µm Ø). RMVs, microvesicles of RBC origin, were gated as CD235a (glycophorin A) positive events within the microvesicle gate. Annexin V (phosphatidylserine) and CD47 were gated within the RMV gate. RMVs per µL supernatant was calculated as: (number of events CD235a / number of events TruCount beads) × (number of beads in tube / sample volume). Annexin V and CD47 positive RMVs (%) were calculated as number of positive events / total number of RMVs.

FlowJo v.10.3 or later (Ashland, OR, US) was used for RMV computation.

ATP (µmol/g Hb)

250 µL RCC sample was added to 250 µL 12 % trichloroacetic acid + 50 µL 0.1 M EDTA in a glass tube (length 100 mm, Ø 16 mm). The tube was vortexed and incubated for 15 minutes on ice. After incubation, the tube was vortexed and 11 µL of the mixture was pipetted to Ellerman tube 1 pre-filled with 500 µL Tris-EDTA (BioThema, Handen, Sweden). Ellerman tube 1 was vortexed and 5 µL each of the mixture was pipetted to Ellerman tube 2a and 2b (duplicates) pre-filled with 400 µL Tris-EDTA. Ellerman tubes 2a and 2b were stored at ≤ −70 °C until analysis.

The samples were prepared for luminometry analysis using ATP Kit SL (BioThema, Handen, Sweden) according to the manufacturer's instructions, and then read on Orion Microplate Luminometer (Berthold, Pforzheim, Germany).

2,3-DPG (µmol/g Hb)

500 µL RCC sample was added to 2,5 mL 0.6 M perchloric acid in a glass tube (length 100 mm, Ø 16 mm). The tube was vortexed and centrifuged at 2152 × *g*, 8 minutes, 10 °C. 2 mL supernatant was pipetted to a new glass tube and 250 µL 2.5 M K_2CO_3 was added. The tube was incubated on ice/at 10 °C for ≥ 60 minutes. After incubation, the tube was centrifuged again at 2152 × *g*, 8 minutes, 10 °C. 500 µL supernatant was pipetted to each of two Ellerman tubes. The two Ellerman tubes were stored at ≤ −70 °C until spectrophotometric analysis.

The samples were prepared for spectrophotometric analysis using 2,3-DPG test kit 10148334001 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions, and then read on Jenway 6500 Spectrophotometer (Barloworld Scientific Ltd., Dunmow, Essex, UK) (**Papers I and II**) or SpectraMax (Molecular Devices, Sam Jose, CA, USA) (**Paper IV**).

3.3.2 Additional analyses

Residual leukocytes (count per unit)

Residual leukocytes were counted with ADAM-rWBC Residual Leukocyte Counter (NanoEnTek, Seoul, South Korea). In short, 100 µL sample + 400 µL r-Solution from the ADAM-rWBC kit were mixed in an Eppendorf tube and100 µL of the mixture was pipetted to an r-Slide cuvette. After 4–7 minutes of sedimentation, the cuvette was read in ADAM-rWBC.

Bacterial screening (colony forming units; CFU)

Bacterial screening was performed to ensure that there was no contamination of bacteria that impacted the RCC quality. All RCCs were tested for sterility after the last day of storage lesion analysis. The bacterial screening was performed at Clinical Microbiology, Karolinska University Hospital. < 5 CFU/mL is considered negative.

Osmolality (mOsm/kg)

Osmolality was measured to determine the quality of the washing process post-deglycerolisation. SAGM has an osmolality of 376 mOsm/kg[27]. A value far above this is considered an indication on residual glycerol, which might impact the RCC quality[36,108,144]. In **Paper I**, supernatant was prepared via the haemolysis centrifugation protocol, after which the osmolality analyses were performed at Clinical Chemistry, Karolinska University Hospital.

Albumin (g/unit)

Albumin was measured to determine the efficacy of the washing procedure. As albumin comprises 50–60 % of the total protein in healthy donors[145], albumin can be considered an indirect indicator of total protein content (total protein content should be < 0.5 g/unit in washed RCCs[13]). In **Paper IV**, supernatant was prepared via the haemolysis centrifugation protocol, after which the albumin analyses were performed at Clinical Chemistry, Karolinska University Hospital.

IgA (mg/L)

IgA was measured to determine the efficacy of the washing procedure. There are no common European specifications for IgA in washed RCCs, but the Association for the Advancement of Blood & Biotherapies (AABB) stipulates < 0.5 mg/L in the US[103]. In **Paper IV**, supernatant was prepared via the haemolysis centrifugation protocol. IgA in the pool (pre-intervention) was analysed by turbidimetry (Optilite, Binding Site, Birmingham, UK) whereas IgA post-intervention was measured using an inhouse enzyme-linked immunosorbent assay (Clinical Immunology, Karolinska University Hospital).

3.4 STATISTICAL METHODS

Paper I

We chose not to assume a Gaussian distribution because of the small sample size (SU1 and 2: n = 8 day 1–4; SU3: n = 4 day 7–10; WU: n = 4 day 1–10). Therefore, the data comparisons of **Paper I** were assessed non-parametrically, using Mann-Whitney test for statistical evaluation. The data was presented as median with interquartile range (IQR). A p value of < 0.05 was considered statistically significant. GraphPad Prism v.8.0 for Windows (GraphPad Software Inc., La Jolla, CA, USA) was used for computation.

Paper II

Gaussian distribution was verified by D'Agostino-Pearson normality test, and two-way analysis of variance (ANOVA) was used to test statistical significance between the four RCC study arms (n = 16 per study arm). Holm-Šídák's correction for multiple comparisons was added as post-hoc test. Fisher's exact test was used for contingency test of 2,3-DPG at day 21. All data was presented as mean ± standard deviation. $p < 0.05$ was considered statistically significant. GraphPad Prism v.8.2 for Windows was used for computation.

Paper III

Gaussian distribution was verified by D'Agostino-Pearson normality test. Two-way ANOVA was applied to test statistical significance between the four study arms at different points of measurement, whereas repeated measures one-way ANOVA was used to test differences between points of measurement within a single study arm. Holm-Šídák's correction was used as post-hoc test. All data was presented as mean ± standard deviation. $p < 0.05$ was considered statistically significant. GraphPad Prism v.8.2 for Windows was used for computation.

Paper IV

D'Agostino-Pearson normality test confirmed a Gaussian distribution, which justified data presentation via mean ± standard deviation. Repeated measures two-way ANOVA with ad-hoc test Holm-Šídák's multiple comparisons were used to test significance was between the four study arms. Differences between sampling occasions within a single study arm was assessed by paired t-test. For 2,3-DPG, day 2 and day 7 were tested with paired t-test whereas a two-way ANOVA was used for day 14. $p < 0.05$ was considered statistically significant. GraphPad Prism v.9.3 for Windows was used for computation.

3.5 ETHICAL CONSIDERATIONS

Ethical permits are rarely required for the type of studies that are included in this thesis, because of the anonymised material and lack of public traceability back to the donor. These principles are based on the European Commission directive 2002/98/EC[146], setting standards of quality and safety for the collection, testing, processing, storage and distribution of human blood and blood components. These standards are applied independent of whether the material is used for patients or research.

Nevertheless, we conducted an ethical permission application for **Paper II**, because of its size, company involvement, and because of regulatory requirements from Macopharma, provider of the utilised blood bag systems. It was submitted to EPN, Regionala Etikprövningsnämnden (Regional ethical committee), Stockholm, that decided not to try it further. The decision (2018/1098-31) was based on that the biological material could not be traced back to the participating donors, and therefore the study was not applicable to the law (2003:460) about ethical permission for human research. However, they stated that they had no ethical objections against the study.

None of our studies imposed an increased risk for the donors. At donation, a standardised volume of 450 mL ± 10 % was collected, which is considered safe for the donors. Only donors that were already accepted for ordinary blood donation were considered as study donors. The blood donation procedures are documented in the Karolinska blood establishment quality management system, and regularly subject to inspection by IVO (Inspektionen för vård och omsorg, Health and Social Care Inspectorate) and Läkemedelsverket (Swedish Medical Products Agency). In addition, the donors did not note a difference when donating for the studies, as the material was already in use for regular blood donation (for the DEHT bags of **Papers II and III**, the needle was still CE marked and used in the conventional blood bag systems NPT6280LE, Macopharma).

4 RESULTS AND DISCUSSION

This chapter provides an overview of the key findings and considerations of the studies included in this thesis. Full details are provided within each individual paper **(Papers I–IV)**.

Paper I

In **Paper I**, it was demonstrated that it was possible to successfully produce paediatric-sized, cryopreserved RCCs with satisfactory quality. Key processing steps included:

- Split of a full-sized glycerolised RCC into three parts, which were frozen in similar bags (EVA-bags, nominal volume 1940 mL) as the full-sized RCCs. This enabled washing with the ACP 215 without encountering flow occlusion, which was the case in our pre-study pilot trial with smaller bags.
- A one-hour rest in RT between the deglycerolisation and manual washing procedure, which was hypothesised to reduce the overall RBC stress load.
- Supernatant reduction of the deglycerolised/washed split RCCs, to adjust the post-wash haematocrit to 60 %.
- Transfer of the final deglycerolised/washed split RCC to a 150 mL paediatric storage bag to reduce the RCC volume-to-bag surface ratio during storage.

The study results suggested that split RCCs would be possible to store for at least as long as non-split RCCs (referred to as 'whole units' in **Paper I**) after cryopreservation and thawing. This conclusion was primarily based on that split RCCs had lower haemolysis and extracellular K^+ concentration than non-split RCCs throughout the ten assessed days ***(Figure 14)***. RCCs commonly have a post-cryopreservation/thawing shelf-life of 4–7 days to not risk exceeding 0.8 % haemolysis[13]. Encouragingly, as neonates are extra sensitive to excessive loads of Fe^{3+} and K^+, none of the split RCCs exceeded this limit even on day 10 post-thawing, whereas one of the non-split RCCs did.

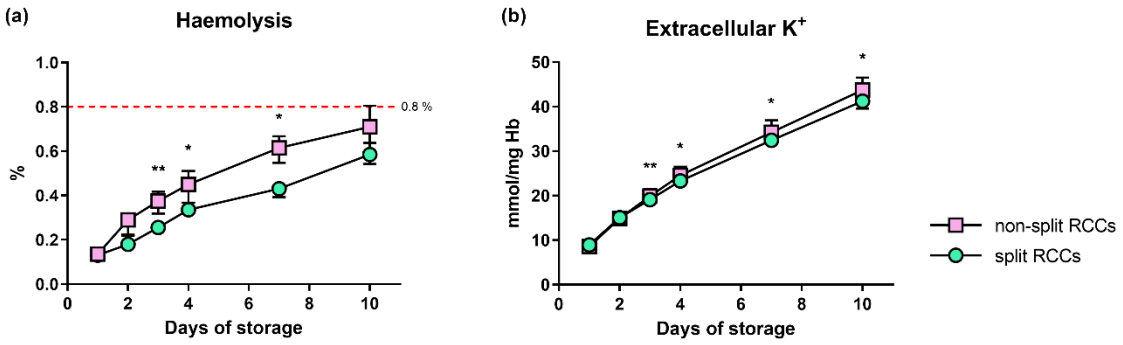


***Figure 14**. (a) Haemolysis and (b) the concentration of extracellular potassium ions (K^+) were lower in split RCCs from storage day 3 onwards. Data are presented as median with interquartile range. Significance levels: * = p < 0.05, ** p < 0.01. Red dashed line: EDQM haemolysis limit (0.8 %)[13].*

Compared to a conventional RCC, pH was substantially lower after cryopreservation; the median was 6.26 in split RCCs and 6.39 in non-split RCCs (pH is approximately 7.0 in a fresh RCC stored in SAGM additive solution[31], as demonstrated in **Papers I**–**IV**). The low pH was likely a consequence of the removal of residual plasma during the cryopreservation/washing procedure, as plasma normally has a buffering effect on pH (a significant pH reduction was also seen after the plasma was removed during the PR and automated washing procedure in **Paper IV**). Considering the low pH, it was not surprising that the RBC metabolism was impeded, as visualised through the low ATP values that, especially for the split RCCs, lacked the initial characteristic concentration increase[31,108].

Normally, decreased metabolism rate and impeded ATP generation lead to increased storage lesion and ultimately haemolysis. In addition, the split RCCs were exposed to more extensive mechanical stress than the non-split RCCs. According to these rationales, it would have been expected that the haemolysis was higher in the split RCCs than in the non-split RCCs. However, such a relationship could not be established. Other studies on cryopreserved RBCs have demonstrated a similar discrepancy between haemolysis and metabolism, confirm that the premature haemolysis is probably a function of osmotic and mechanic rupture rather than being storage-related[36,38,108]. However, since cryopreserved RBCs are very brittle, the better preservation of the membrane integrity in our split RCCs, despite exposure to more mechanical stress than corresponding non-split RCCs, was still unexpected.

It appears as if the differences in storage media, i.e. haematocrit and supernatant composition, had a substantial impact on the results. In the non-split RCCs, the median haematocrit was 45 %, meaning that the RCC contained quite a lot of volume of storage solution and residual washing solution (calculations estimated the SAGM content of the supernatant to approximately 75 %). In the split RCCs, where new SAGM was added after supernatant extraction, the SAGM content was approximated to > 90 %. The benefits of continuous suspension of RBCs in SAGM instead of NaCl even during short-time events such as a washing process has been demonstrated[19]. Possibly, these advantages were reflected also in the results of **Paper I**. The importance of the storage media composition may also have been enhanced since pH was too low to allow an effective metabolism in neither the split nor the non-split RCCs. It may also be argued that in **Paper I**, storage lesion was measured for 10 days only, which is a considerably short period and leaves little time for storage-dependent metabolic changes. Furthermore, the RBCs in the split RCCs spent less time in contact with liquid glycerol because of their smaller volume, which shortened the freezing and thawing time compared to non-split RCCs. The shortened contact time may have reduced the overall haemolysis generation, as a connection between increased haemolysis and the cryopreservation steps involving liquid glycerol has been previously demonstrated[38]. However, even in light of these arguments, low pH and ATP levels are detrimental for the RBC integrity. Therefore, the lower haemolysis and extracellular K^+ in the split RCCs weighs even heavier when considered in this context.

Our method has several advantages for paediatric care. In addition to reducing the exposure to harmful substances such as free Fe^{3+} and K^{+}, the tailored haematocrit of the split-RCCs decreases the risk of hyperhydration. The possibility to thaw smaller portions of RCCs of rare blood types on demand both reduces the number of involved donors at repeated transfusions and increases the availability of rare blood. This contributes to ensuring an overall sufficient blood supply.

Paper II

The overall conclusion of this comparative study of DEHT vs DEHP blood bags, is that storage in DEHT did exhibit minor disadvantageous effects on the RBC membrane, but not as severe as may have been expected. In a previous study executed at the Karolinska blood establishment, where RBCs were stored in a polyolefin blood bag completely free of plasticizer, the mean haemolysis exceeded 0.8 % already after 3–4 weeks when using PAGGSM as additive solution and after 4–5 weeks when using the experimental phosphate-adenine-glucose-guanosine-gluconate-mannitol (PAGGGM)[129]. In the study presented in **Paper II**, the haemolysis during DEHT storage remained well below the allowed limit throughout the entire 49 days assessment ***(Figure 15)***; in fact, haemolysis for the DEHT-stored RBCs resembled "normal" DEHP storage[31]; 0.29 ± 0.03 % in DEHT/SAGM and 0.22 ± 0.02 % in DEHT/PAGGSM at day 42 (corresponding DEHP storage in this study was even lower).

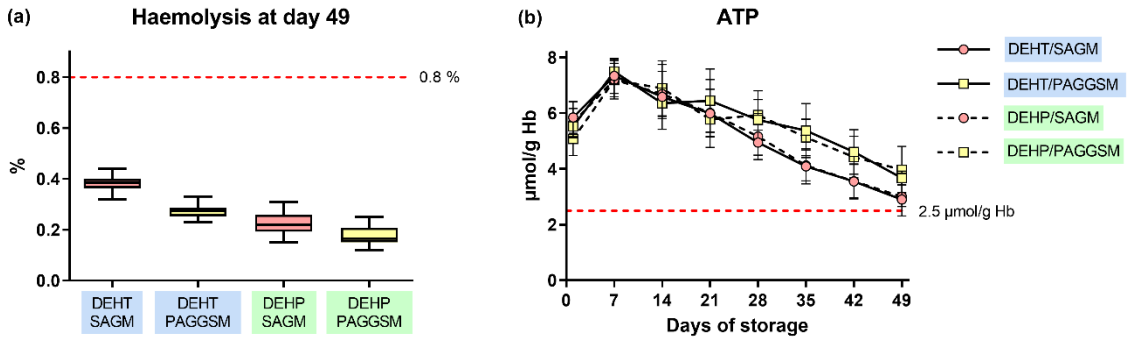


Figure 15. *(a) Haemolysis was well below the EDQM limit (0.8 %; red dashed line)[13] in DEHT storage throughout 49 days of storage, independent of additive solution. (b) Choice of plasticizer had no impact on ATP preservation, however, PAGGSM additive solution was superior to SAGM for RBC storage (2.5 µmol/g Hb, corresponding to a 75 % 24-hour survival in the circulation[79]; dashed red line).*

Similar to haemolysis, absence of DEHP resulted in increased RMV generation, as did storage in SAGM, resulting in the highest count in DEHT/SAGM and the lowest in DEHP-PAGGSM. However, compared to haemolysis, where significant differences were discernible immediately after storage start, the RMVs generated from DEHT storage only differed from the DEHP study arms from day 21 in DEHT/SAGM, whereas no significant difference could be demonstrated at all between DEHT/PAGGSM and the DEHP arms up to and including day 42; current shelf-life end point in Sweden.

When considering extracellular K^+ and 2,3-DPG, storage lesion was actually less pronounced in DEHT storage compared to DEHP, regardless of choice of additive solution. Especially the 2,3-DPG results were compelling, as at day 21, 34 % of the DEHT-stored RCCs were depleted of 2,3-DPG whereas corresponding number in DEHP was almost the double; 62 %. No difference could be attributed to additive solution for neither extracellular K^+ nor 2,3-DPG.

The metabolic factors, except for extracellular K^+ that reflects both metabolism and membrane integrity deterioration, were not affected by plasticizer. However, better ATP preservation, slower generation of lactate, lower percentage of RMVs expressing phosphatidylserine and reduced cell swelling as measured by MCV together indicated that PAGGSM is a superior storage media for both DEHT and DEHP storage ***(Figure 15)***.

The plasma and platelet assessments in **Paper II** were in line with previous research concluding that DEHP removal is foremost a challenge for RBC storage[29].

Based on the next to "normal" haemolysis values during DEHT storage, it may be hypothesised that DEHT may still provide some membrane protection for the RBCs, although not at distinct as DEHP. DEHT does leak from the PVC matrix as well, although in far less amounts than DEHP[136]. However, compared to our previous study on poleolefin storage, the sampling technique has been refined, and in addition, the poleolefin blood bag system was based on WB filtration whereas the DEHT study utilised buffy coat removal and subsequent RBC filtration as processing strategy. It would be very interesting to compare storage in polyolefin and PVC-DEHT during identical conditions, to determine whether DEHT possesses some amount of membrane protective properties or not.

The most important take home message of **Paper II** is that, first of all, DEHT is a viable plasticizer option for RBC storage after the DEHP-ban comes into effect. The second message is that RBC membrane damage can be mitigated, independent of plasticizer and additive solution choice, by using adequate processing and sampling techniques that reduces the overall exposure to mechanical stress. The routine processing techniques of the Karolinska blood establishment include collection-day WB separation, RBC leukofiltration and comparatively low centrifugation *g*-force, which are all known to be favourable for stress reduction[14,112,147,148]. Very likely, when moving to DEHP-free RBC blood bags, it will be advantageous to not only consider new additive solution compositions, but also re-assess workflows and processing strategies.

Paper III

The results of **Paper III** reminisced those of **Paper II** to a great extent, however, a considerable impact of the irradiation damage to the RBC membrane was evident.

The parameters directly reflecting RBC membrane damage, i.e. increased haemolysis and microvesicle count, were more elevated than corresponding non-irradiated results in **Paper II**. Additionally, the increase had earlier onset than during non-irradiated conditions ***(Figure 10)***. However, the conclusion from **Paper II** remained: DEHP outperformed DEHT and PAGGSM

outperformed SAGM for RBC storage quality, so by choosing PAGGSM over SAGM, any adverse consequences from storing the RBCs in a DEHP-free blood bag could be mitigated.

No significant differences could be demonstrated between the plasticizers or additive solutions in neither haemolysis nor RMV at day 14 post-irradiation. This implies that, at least when irradiation is performed early during storage, a reduction in shelf-life as a consequence of the DEHP removal may perhaps not be necessary for irradiated RCCs in countries that allow maximum 14 days post-irradiation shelf-life, such as Sweden. Nonetheless, since there is a demonstrated relationship between time of irradiation and generation of haemolysis[49,105], it would be beneficial to complement this study with a "worst-case scenario study" on irradiated RCCs stored in PVC-DEHT, where the pre-irradiation time span is longer.

Extracellular K^+ did differ slightly to the non-irradiated RCCs in **Paper II**: In **Paper III**, the SAGM/DEHT combination exhibited small but significantly higher concentrations than both PAGGSM arms after irradiation. However, the differences were minor, suggesting a neglectable clinical impact. Even so, it was again demonstrated that combining DEHT with PAGGSM was an efficient mitigation strategy against excess K^+ leakage. Except for extracellular K^+, no other of the measured metabolic markers differed in a way that could be related to choice of plasticizer; only additive solution. Here, it was shown that SAGM seemed to instigate an overall higher metabolism rate.

No difference linked to additive solution was detected for the externalisation of RMV phosphatidylserine. Instead, the percentage of phosphatidylserine positive RMVs increased over time in all study arms, which, in the paper, was suggested to be connected to the oxidative stress generated by the irradiation process. The comparable increase for all study arms could possibly be explained by that the impact of irradiation triumphed that of choice of plasticizer and additive solution. Interestingly, the increase of phosphatidylserine was in contrast with the similar irradiation procedure performed in **Paper IV**. This is discussed further in that section.

Paper IV

The results in **Paper IV** indicated that PR-treated RCCs possess adequate quality to allow storage for 42 days post-collection; one week longer than what has previously been commercially approved, and similar to conventional RCCs in Sweden.

PR-RCCs exhibited similar haemolysis levels to the conventional, reference RCCs throughout the storage period ***(Figure 16)***, and in addition, slightly slower RMV generation rate. The latter may have been a function of the included washing step that likely disposed of the most fragile RBCs (a similar principle as the split RCCs in **Paper I**)[38], or the PR-induced increased ATP concentration, or a combination of both.

Substantially more membrane damage was detected after irradiation and automated washing as compared to after PR. This was demonstrated as faster haemolysis, RMV and extracellular K^+ increase rates ***(Figure 16)***. In irradiated RCCs, this was expected, considering the strong onset of ROS instigated by radiation[45,46]. Automated-washed RCCs are subject to additional

mechanical stress through the washing procedure, however, the stress also likely ruptured the most fragile cells which would then have been washed away in the supernatant exchange step, as indicated by the decline in RMV count directly post-washing. Evidently, the mechanical stress load of automated washing outweighs the potential benefits of removal of fragile cells/accumulated RBC debris and subsequent addition of new additive solution.

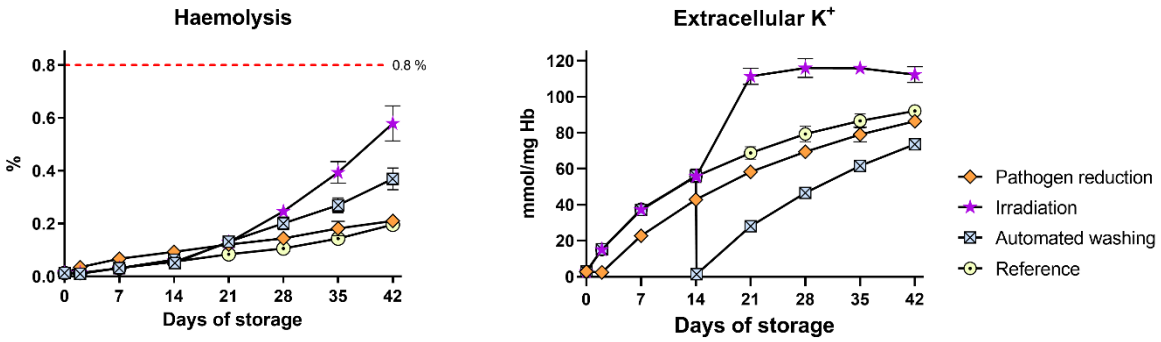


***Figure 16**. The membrane damage, visualised through (a) haemolysis and (b) extracellular potassium ion (K^+) concentration, was much more pronounced after irradiation and automated washing than after pathogen reduction. Red dashed line: EDQM haemolysis limit (0.8 %)*[13].

There were metabolic similarities between PR treatment and the effects seen by PAGGSM usage in **Papers II and III**. After PR treatment, less glucose was available and pH declined approximately 0.15 units compared to the reference RCCs. This led to a slower lactate accumulation. Similar figures and consequences were observed when RBCs were stored in PAGGSM. Simultaneously, ATP increased substantially more after PR than in the reference study arm ***(Figure 6)***. The ATP peak concentration in SAGM and PAGGSM storage did not differ significantly, but ATP decreased slower in PAGGSM-stored RCCs. It is often argued that storage solutions with an alkalic pH are beneficial for RBC metabolism, and that an acidic environment is the RBC's worst enemy[22,26,27,149]. While this may be true, the combined results from our studies indicate that a more acidic pH at storage start is not necessarily linked to a more acidic pH at storage end and, as such, suggest that more factors than pH may be involved in driving the RBC metabolism. Furthermore, there seems to be a delicate balance between the benefits and disadvantages of a decreased pH; low pH will inhibit the glycolysis rate, which in turn reduces the rate of ATP generation but also lactate accumulation.

The PR process was slightly less efficient for plasma removal than automated washing, but to be fair, the design of the current PR protocol has been designed primarily for efficient removal of amustaline metabolites. Possibly, the PR centrifugation protocol can be optimised also for plasma removal, either by adjustment of individual parameters or by adding an additional centrifugation cycle, which has been demonstrated to enhance plasma removal in both manual and automated washing procedures[19,150]. However, again, adding additional mechanical stress should be approached with caution, but if successful, an optimised PR centrifugation protocol would be a valuable improvement in order to consider PR-RCCs as a qualified replacement

option to washed RCCs. An additional benefit of replacing automated washing with PR is that less haemoglobin is lost during PR processing, which better maintains the haematocrit of the RCC and reduces the risk of transfusion-induced hyperhydration.

On the downside, PR-RCCs demonstrated a premature depletion of 2,3-DPG compared to the other study arms ***(Figure 6)***, which makes the use of PR-RCCs for neonates or in situations of trauma/excessive bleeding, where an optimal oxygen delivery capacity may be more pivotal than in top-up transfusion, subject for further studies.

Paper IV also addressed the complexity of assessment of RMVs. A different centrifugation protocol for supernatant preparation compared to the previous papers of this thesis first of all generated a more than 10-fold higher count of RMVs. In **Papers I**–**III**, following a protocol recommended by an international collaborative workshop[151], a large amount of RMVs were likely washed away in the second centrifugation cycle. When the protocol was changed to single centrifugation in **Paper IV**, a significantly higher amount of RMVs was counted. We also observed differences in surface structure characteristics as a consequence of the differences in centrifugation protocol. For instance, the percentage of externalised phosphatidylserine increased after irradiation in **Paper III**, whereas correspondingly, the irradiated RBCs decreased most of all study arms in **Paper IV**. Supernatant preparation and flow cytometry protocols vary largely between different blood establishments, which makes inter-blood establishment comparisons problematic. An international standardisation of the RMV analysis protocol would greatly enrich the value of using RMVs as a measure to understand the impact of RBC storage lesion.

The results of **Paper IV** support previous rationales warranting shelf-life reduction after irradiation and washing of RCCs, as a measure to maintain storage lesion-related safety margins. In addition to the original purpose of PR; increased blood safety in terms of infectious diseases, the introduction of a non- shelf-life reduced PR component as a substitute to both irradiated and washed RCCs would likely help reducing the outdating rate and decrease stressful on-demand processing; thereby facilitating stock-keeping of RBCs for irradiated and washed indications.

However, it must be emphasised that the effects of PR on storage lesion presented in **Paper IV** does not apply to all systems for PR. In studies using corresponding technology from other manufacturers, strongly elevated haemolysis levels and corresponding additional evidence of excessive storage lesion progression have been demonstrated[67-69]. This highlights the importance of individual assessments of each system presented for future market use.

5 CONCLUSIONS AND POINTS OF PERSPECTIVE

The RBC blood supply is facing a number of oncoming challenges, including the DEHP-ban, the growing risk of emerging pathogens, the imbalanced equation of a decreasing donor/increasing patient population and the increasing need to stock-keep rare blood types. The COVID-19 pandemic, recurrent extreme weather and political instability have together actualised the need to increase the preparedness and ensure a safe and sufficient blood supply. One important cornerstone in this quest is to avoid a decrease in RBC quality and shelf-life. A decreased quality or shorter shelf-life may increase wastage, which is very contra-productive in a situation where the donor population is already declining.

The four individual studies included in this thesis provide four different ways of assessing the same challenge: stress, whether mechanical, osmotic, from irradiation or by removal of membrane stabilizing elements such as DEHP, is detrimental to the RBC integrity and survival. This thesis shows that there are ways to balance the stress and counteract the possible deleterious consequences without taking the "easy way out" and shorten the shelf-life of the RBC component.

Shortening the shelf-life is sometimes a necessary measure to ensure patient safety, such as the case has been historically with irradiated and washed RCCs. However, shelf-life shortening is also a quick fix to solve a problem, which has positive short-time consequences but does not necessarily provide the best long-term solution for the overall blood supply. Especially in current times, where there is a universal call for strengthening the blood supply, and the EDQM "Guide to the preparation, use and quality assurance of blood components" guidelines are proposed to be referred in legislation[9,13], it is advantageous to look at other, more sustainable alternatives.

Shelf-life not only has an effect on wastage, but also on the availability of blood components. Because of the risk of outdating, many smaller hospital blood banks choose to order irradiated and washed RCCs on demand, rather than keeping a local supply. This instead implicates a risk for the patient, who may have to wait for proper blood if the situation is urgent or the transports are delayed. The opposite practice, keeping irradiated and washed blood in stock and issuing them to patients not in need of these secondary processing measures to avoid outdating, is also a suboptimal strategy. As demonstrated in this thesis, the haemolysis and the RBC debris are significantly higher at the last day of storage in irradiated and washed components than in conventional RCCs.

In this thesis, it has been shown that it is possible to outsmart the option of shelf-life reduction. By understanding and applying long-standing rationales about the RBC biology and how RBCs are impacted by blood banking, as well as new expertise gained from the included studies, efforts were not only made to analyse the storage lesion, but also to limit it as much as possible. RBC preservation was the primary focus of all four papers; it was assessed how the RBC storage lesion was impacted by a new plastic material for blood bags, how inactivation technology for pathogens can be applied in a broader perspective, and how cryopreserved

RBCs may be tailored for paediatric patients, while simultaneously mitigating storage lesion by choosing appropriate processing strategies. The use of such strategies may hold an even more important role in the future, when more personalised treatment strategies are called for and the DEHP-ban is taken into effect. Even today, conventional RCCs sometimes exceed the maximum allowed haemolysis limit due to mishandling during processing/storage or donor-related issues. Without DEHP, the margins will probably be smaller.

"Automation" has been considered a key concept in blood processing during the last two decades. On the manufacturer side, there have been large efforts in the development of different automated features on processing equipment; both for WB separation and for specific secondary processes such as platelet processing and component washing. The idea and driver behind automation is that a standardised process is easier to manage and, by that, errors introduced by the human factor will be easier to avoid and the overall quality will be less susceptible to individual variations.

While this may be a fair argument for automation, not all consequences of automation are desirable. With a locked process, it is more difficult, and sometimes even impossible, for necessary individual adjustments. The ACP 215 is an example on that automation can be both an advantage and a disadvantage: the mean output volume of the washed RCC differs very little for all washed units, independent of the haematocrit of the initial RCC. Because of this, the RCC often becomes unnecessarily diluted, and the haematocrit is lower than optimal[36,108] This has previously excluded the use of ACP215 for paediatric RCCs. To circumvent this limitation, we had to add a manual centrifugation step to concentrate the RCC component after finished washing procedure.

Lately, there has been a general renewed interest in manual processing procedures, not just because they are controllable, but also because evidence is accumulating that they impose less stress onto the cells. It has been demonstrated that washing of RBCs with a method that includes double manual centrifugation of RBCs is less deleterious to the cell membrane than washing with the automated ACP 215[19]. Likewise, higher haemolysis and free K^+ was found after processing with the automated, multi-component WB separation device Reveos, when it was compared to traditional component processing where centrifugation and extraction are separated[152]. A similar relationship has been demonstrated for platelet processing, where platelets have exhibited increased activation after automated processing compared to manual[153-155]. Because of the improvement in quality and function, in combination with the optimised compatibility with technology for PR of platelet concentrates[156], a renaissance in manual platelet processing has been seen throughout Europe. In a time where there is an increased focus on precision medicine and individual adaptability, as well as an urgency to optimise the RBC storage quality, it would not be surprising if a gradual progression into more manual processing becomes the new "back to the future" strategy. Also, concerning PR, its original purpose must not be forgotten. It is not possible to predict what the characteristics of the next pandemic will be, and the addition of this extra safety layer is very well in line with the increased urgency for blood supply contingency and emergency preparedness.

Regardless of any novel or revisited strategy to increase the RBC storage quality or supply safety, everything we know so far needs to be re-explored in the context of a DEHP-free setting. Until properly evaluated, we will not be able to assume that old "truths" about RBC storage lesion from PVC-DEHP can simply be extrapolated to a different plasticizer. Although early studies suggest that a conversion to plasticizers such as DEHT or DINCH will be feasible provided that membrane preserving measures are taken and the storage lesion is kept at bay, these studies are predicted to only be the start of a very extensive era of validation work. In short, all RBC components downstreams of the unseparated WB need to be re-validated in non DEHP-material. In addition, the blood bag manufacturers may choose different plasticizers and/or additive solutions, meaning that every RBC component may require multiple parallel validations to ensure that all combinations are properly investigated and provide sufficient RBC quality.

A key strategy to manage an effective non-DEHP transition within the narrow time frame of the DEHP sunset date is collaboration; between blood establishments, manufacturers and regulatory oversight bodies. A working group within the European Blood Alliance recently proposed a framework for sharing of validation data between blood establishments (publication in progress). This framework includes rationales and guidance for the validation set-up to accommodate to the quality requirements of most jurisdictions, and thereby allow the adoption of results obtained outside of the own blood establishment, or even country. In a corresponding multi-scenario perspective, the EDQM has developed recommendations and guidance tools to ensure blood supply continuity in a number of emergency-related key risk scenarios[10]. Although predictable, the non-DEHP transition could very well impose a substantial threat to the blood supply, if not addressed pro-actively by all involved stakeholders already today. Common with the EBA framework, the EDQM recommendations emphasise the urgency for increased collaboration and cooperation as a key measure.

In conclusion, RBCs are facing a series of coinciding challenges that will likely enhance the importance of mastering the fine art of processing. In order to ensure a sustainable, safe and high-quality blood supply, we will most probably need to reconsider both our processes and how we view them. Likely, the establishment of a new perspective on both national and international cooperation will be required. This may be a favourable contribution also for the management of future challenges.

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