Importance of donor factors and whole blood processing in the storage injury of red blood cells

by

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Abstract

Background:

The processing and storage of blood components prior to transfusion are key parts of the blood banking industry. Recently, however, it has been suggested that the long term storage of red blood cells for transfusion could have detrimental clinical implications. In this study we investigate the pre-storage factors, such as donor characteristics and component processing conditions, that influence the way red blood cells change and accumulate injury during storage.

Materials and Methods:

Retrospective analysis of large scale blood bank databases was performed to identify sources of variability within stored red cell concentrates. Prospective storage studies were designed to further assess the effects of different pre-storage variables based on the observations of the retrospective data analysis. The volume of plasma and additive solution was measured in red cell concentrates processed using different methods; red blood cells were then stored in known volumes of plasma and additive solution to assess the impact on red cell storage injury. To investigate red blood cell turnover and senescence, red blood cell size and density was measured in stored red cell concentrates from different donor groups.

Results:

Component processing conditions and donor factors were both found to influence the post-storage characteristics of stored red cell concentrates. Buffy-coat component processed units exhibited less storage injury than units processed using the whole blood filtration method. The influence of component processing was found to be independent of the residual plasma volume and additive solution volume. Units from female donors exhibited less storage haemolysis than units from male donors, and increasing donor age was also associated with increasing storage haemolysis. Donor sex and age were shown to influence; pre-donation haemoglobin concentration, haematocrit, mean cell volume, cell density, cell haemoglobin content. Young female donors exhibited the lowest pre-donation haemoglobin concentrations, and the largest, least dense red blood cells.

Conclusions:

The effect of hypothermic storage on red blood cell characteristics is dependent not only on the duration of storage; but also the component manufacturing conditions the cells are exposed to, and the unique characteristics of the donor's blood. Understanding how these variables generate definable populations of red cell products is a key step in providing the safest, most appropriate transfusion components to hospitals and patients.

Preface

Portions of this thesis have been published as journal articles. Chapter 2 has been published as; A. Jordan, D. Chen, Q. –L. Yi., T. Kanias, M. T. Gladwin, J. P. Acker, "Assessing the influence of component processing and donor characteristics on quality of red cell concentrates using quality control data." *Vox Sanguinis*, 2016; 111(1):8-15. I was responsible for the construction of the database, study conception/design, data analysis and construction of the manuscript. D. Chen was responsible for data analysis and assisted with preparing the manuscript. Q. –L. Yi was responsible for statistical analysis. T. Kanias was responsible for study conception/design. M. T. Gladwin was responsible for study conception/design. J. P. Acker was responsible for study conception/design, construction of the database, data analysis and construction of the manuscript. All authors critically reviewed the manuscript. Appendix A has been published as; A. Jordan, J. P. Acker, "Determining the Volume of Additive Solution and Residual Plasma in Whole Blood Filtered and Buffy Coat Processed Red Cell Concentrates." Transfusion Medicine and Hemotherapy, 2016; 43:133-136. I was responsible for study conception/design, data collection, data analysis and construction of the manuscript. J. P. Acker was responsible for study conception/design, data analysis and construction of the manuscript.

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List of Abbreviations and Symbols

Abbreviations

1,3-DPG	–	1,3-diphosphoglycerate
2,3-DPG	–	2,3-diphosphoglycerate
ABLE	–	Age of blood evaluation
AS-1	–	Additive solution 1 (Asdol, Baxter)
AS-3	–	Additive solution 3 (Nutricel, Pall Medical)
AS-5	–	Additive solution 5 (Optisol, Terumo)
ATP	–	Adenosine triphosphate
BC	–	Buffy coat
CI	–	Confidence interval
CPD	–	Citrate phosphate dextrose
GLUT-1	–	Glucose transporter 1
Hb	–	Haemoglobin
Hct	–	Haematocrit
HLA	–	Human leukocyte antigen
Hly	–	Haemolysis
HNA	–	Human neutrophil antigen

HSL	–	Hypothermic storage lesion
LR	–	Leukoreduced
lysoPC	–	Lysophosphatidylcholine
metHb	–	Methaemoglobin
MCH	–	Mean cell haemoglobin
MCHC	–	Mean cell haemoglobin concentration
MCV	–	Mean cell volume
NADPH	–	Nicotinamide adenine dinucleotide phosphate
NO	–	Nitric oxide
PS	–	Phosphatidylserine
QC	–	Quality control
QMP	–	Quality management program
RECESS	–	Red cell storage duration study
RBC	–	Red blood cell
RCF	–	Relative centrifugal force
RCC	–	Red cell concentrate
Rh	–	Rhesus antigen
SAGM	–	Saline adenine glucose mannitol
SD	–	Standard deviation

TRALI	–	Transfusion related acute lung injury
WBC	–	White blood cell
WB	–	Whole blood
WBF	–	Whole blood filtered

Symbols

dL	–	Decilitre
fL	–	Femtolitre
g	–	Grams
g	–	G-force
h	–	Hours
K^+	–	Potassium cation
L	–	Litre
mL	–	Millilitre
mM	–	Millimolar
Na^+	–	Sodium cation
NaCl	–	Sodium Chloride
nm	–	Nanometer
O_2	–	Dioxide molecule (elemental oxygen)

pH	–	Measure of acidity/alkalinity (power of hydrogen)
w/v	–	Weight/volume
Δ	–	Detla (difference between two values)
μm	–	Micrometer
°C	–	Degrees Celsius

Chapter 1

Introduction

Transfusion of blood products is a global practice, effecting millions of patients around the world each year. Ensuring the safety and quality of transfused blood products is the top priority of blood services, and most of the research in the field of transfusion medicine is focused on improving the processes used to collect, manufacture and store transfusable blood products. The following is a review of the current state of red blood cell (RBC) processing, and the relationship between RBC storage and product quality.

1.1. Manufacturing & Storage of Red Cell Concentrates

1.1.1. Red Blood Cell Transfusion

RBC transfusion is a common therapy for the treatment of anaemia and blood loss, in which blood components from a healthy eligible donor are transfused into the patient's circulation. A transfusion is necessary when a patient's blood is incapable of adequately performing its primary function of gas transport; either due to blood loss or disease[1-5]. The roots of transfusion medicine can be traced back throughout history; brought to life through William Harvey's

description of the human circulatory system in 1616[1]. The practices of modern transfusion medicine have come a long way since early attempts at vein to vein transfusions. Over 90 million whole blood (WB) donations are collected annually from over 8000 donation centres worldwide[6]; the need to keep up with the rapidly increasing demand for blood products has been a driving factor behind many of the major developments in blood banking. Although RBC transfusion has become an accepted routine procedure, there are still serious risks associated with it. The safety of blood products has come into question frequently throughout the years, and blood banking practices have been adapted to mitigate some of the major risks associated with transfusion.

Whereas early methods of transfusion involved the transfer of WB from donor to recipient[1, 7], in modern transfusion medicine, a single WB donation is processed into number of transfusable components. Different processing methods are utilised in order to extract additional components such as plasma and platelet components, as well as the RBC component. These components are either transfused, or further processed to produce a wide range of therapeutic products. Separation of components is achieved by fractionating the WB in a centrifuge; the heavy cellular components are separated from the lighter non-cellular components. The composition and distribution of the fractionated components are dependent on the relative centrifugal force (RCF) applied to the WB and the duration of centrifugation. Current WB component separation techniques used by Canadian Blood Services are; the whole blood filtration (WBF) method, which yields a unit of plasma plus a unit of red cell concentrate (RCC), or the buffy-coat (BC) method, yielding an additional buffy-coat component as well as the RCC and plasma. Leukodepletion of red cell components in now standard practice for most national blood services, and is performed by passing the RBCs through a filter prior to storage.

1.1.2. Hypothermic Storage of Red Blood Cells

Early blood transfusions were carried out directly from donor to recipient, as there was no effective means of preventing the blood from coagulating once removed from the body[7]. Direct vein to vein transfusion was a highly unsafe procedure; the risk of thrombosis, embolism and circulatory overload was very high[7]. To address these issues, scientists sought to develop a means of preserving WB in a liquid state long enough to transfuse. Calcium chloride was shown to have a profound effect on blood coagulation, and subsequently it was hypothesised that calcium is essential for the clotting process[8]. Citrate was found to disrupt clotting due to the high binding affinity of calcium for citric acid[8]. The use of citrate as an anticoagulant preserved the blood in a liquid state long enough to be transfused, however the RBCs began to rapidly haemolyse once removed from circulation[8]. Cooling the RBCs to temperatures low enough to slow down cellular metabolism without freezing them was found to slow the rate of cell lysis, however this was only sufficient for short term storage[8-10]. Early storage solutions used simple sugars to further inhibit haemolysis; the first reported storage solution was developed during World War I by Peyton Rous and J.R. Turner, consisting of citrate and glucose[9, 10]. The Rous-Turner solution allowed for the hypothermic storage (4 ± 2°C) of whole blood for up to 4 weeks without significant haemolysis[9, 10]. The ability to collect and store blood safely for transfusion is the basis of blood banking, and in 1917 Oswald H. Robertson established the world's first blood bank in France. Modern storage solutions still share similarities with Rous and Turner's original citrate-glucose solution; however, blood banking practices continued to evolve as our understanding of RBC physiology improved.

Effective storage of RBCs must not only prevent haemolysis, but must also preserve the functionality of the cells once reintroduced into circulation. In order to achieve this, it is important to understand the role of RBCs in gas exchange, and how their physiology is adapted for this role.

The mature RBC is highly specialised for its role of gas transport; a biconcave discoid with a diameter of 7-8 μm, void of a nucleus and any intracellular organelles[11]. The shape of the RBC gives it a large surface area to volume ratio (surface area: 140 μm^2, volume: 100 μm^3), ideal for gas exchange across the cell membrane[12]. The cytoplasm of the mature RBC contains a large amount of haemoglobin (Hb) (95% dry weight, 33% w/v), the protein responsible for oxygen binding[13, 14]. Haemoglobin is a tetrameric globular protein, comprised of two pairs of globin polypeptide chains, and four haem groups, consisting of a protoporphyrin ring and a ferrous iron molecule[13, 14]. Molecules of oxygen are able to bind to the iron molecules of the haem groups, allowing each molecule of haemoglobin to transport four molecules of oxygen, which is taken up in the vasculature of the lungs, and then released as needed in the tissues[13, 14].

Because the RBC extrudes its organelles during development to maximise haemoglobin capacity, the cell membrane is the sole structural component of the mature RBC. The RBC membrane is a highly evolved, complex structure; composed of phospholipids, cholesterol and an array of associated membrane proteins, glycoproteins and glycolipids. There are four primary phospholipids that make up the RBC membrane, forming an asymmetric bilayer structure[15, 16]. Phosphatidylcholine and sphingomyelin are restricted to the outer membrane leaflet, whilst phosphatidylethanolamine and phosphatidylserine (PS) are restricted to the inner membrane leaflet[16-18]. This asymmetry is tightly controlled by a number of energy-dependent phospholipid transport proteins; flippase proteins transport phospholipids from the outer membrane leaflet to the inner leaflet, and floppase protein mediates transport from the inner to the outer leaflet[17-19]. Maintenance of phospholipid asymmetry is vitally important for the RBC survival. Exposure of PS on the outer membrane leaflet of RBC leads to recognition by macrophages and removal by the reticuloendothelial system[19, 20], PS also interacts with endothelial cells causing RBCs to adhere to the walls of the vasculature and disrupt blood flow[19-

21]. Cholesterol in the RBC membrane is evenly distributed across the inner and outer leaflets of the bilayer, forming cholesterol rich membrane domains called lipid rafts[22]. Lipid rafts are dense, cholesterol and sphingolipid rich membrane domains, thought to have important functions in signal transduction, membrane protein sorting and cellular microvesiculation[22-25]. The membrane is the sole structural component of the mature RBC, and as such is responsible for a large number of cellular functions including; transport, cell signalling, and structural and mechanical functions[16]. The cell membrane and underlying cytoskeleton are responsible for the unique structural qualities of the RBC that enable it to effectively perform its primary function of oxygen transport.

As the RBC passes through the microvasculature it must traverse capillaries with a smaller diameter than itself; it is therefore necessary for the cell to deform and then return to its normal shape upon entering a larger vessel[26-28]. Cell deformability is dependent on a number of factors, primarily; membrane elasticity, cytoplasmic viscosity and surface-area to volume ratio[16, 26-28]. Membrane elasticity appears to be affected by a number of factors; the cytoskeleton, and in particular spectrin, is thought to be of critical importance[26-28]. Membrane composition is also believed to play a role in elasticity, specifically cholesterol content. As cholesterol and phospholipids are maintained at equal concentrations in the membrane, it has been suggested that increased cholesterol content reduces membrane deformability[29]. Cytoplasmic viscosity is determined primarily by intracellular haemoglobin concentration, which is maintained within the range of 30-35 g/dL[16, 30]. As haemoglobin is a large protein which cannot be transported in or out of the cell, and mature red blood cells lack the ability to synthesise or break down this protein, the intracellular haemoglobin concentration is controlled through regulation of the cell volume[16, 30]. RBCs maintain their cell volume (average volume = 94 fL ± 14 fL) through regulation of total intracellular cation content using sodium and potassium

membrane transport proteins (sodium-potassium ATPase)[30, 31]. The final important cellular characteristic contributing to cell deformability is surface area to volume ratio, which is governed by the cells shape. The biconcave disk shape of the cell, which is maintained by the cytoskeletal-membrane interactions, gives the RBC roughly a 40% larger surface area compared to a spherical cell of the same volume. The large surface area to volume ratio allows the cell to deform without exerting too much pressure on the membrane, whilst providing a large area for gas exchange[16, 32].

Despite lacking organelles and synthetic equipment, RBCs perform a number of energy dependent processes, primarily the maintenance of membrane asymmetry[19, 20, 33] and the active transport of ions in and out of the cell to maintain cell volume[30, 31]. Red blood cells generate adenosine triphosphate (ATP) through the glycolytic pathway (or Embden-Myerhof pathway), in which glucose is anaerobically broken down into lactic acid[34, 35]. Because the RBC is unable to convert glucose to glycogen for storage, RBCs require a constant supply of glucose in order to generate ATP[34, 35]. *In vivo*, RBCs rely on glucose in the extracellular medium, which is transported into the cell by glucose transporter proteins (primarily GLUT 1) in the RBC membrane[36]. Current generation additive solutions such as SAGM (saline, adenine, glucose, mannitol), and the similar AS-1, AS-3 and AS-5 solutions have been developed to meet the demands of the metabolically active RBC during storage (Table 1.1)[37]. Saline is used as a diluent to reduce the viscosity of the separated RBC component[38, 39]. Adenine is the primary building block of ATP, and is added to increase the total adenylate pool to help maintain RBC ATP levels during storage[39, 40]. Mannitol functions as a free-radical scavenger and membrane stabilizer during storage, and contributes to significantly decreased RBC haemolysis in storage[39, 41]. Despite constant developments and improvements in storage additive solutions, RBC biopreservation is still limited in terms of storage duration and efficacy.

1.2. Quality of Stored Red Blood Cells

1.2.1. RBC Hypothermic Storage Lesion

During RBC storage, the biochemical and morphologic changes occur within the RCC unit are collectively termed the hypothermic storage lesion (HSL). A number of these changes have been identified, and means of mitigating them have been implemented. However, the mechanistic basis for some factors of the storage lesion has not been elucidated, and the causal relationship between different factors is not fully understood. Key biochemical features of the HSL include: depletion of ATP and 2,3-diphosphoglycerate (2,3-DPG), oxidative stress, decrease in pH, influx of sodium (Na^+) cations and efflux of potassium (K^+) cations. Key morphological/biomechanical features of the HSL include: loss of membrane asymmetry, changes in cell shape and the shedding of microvesicles and associated loss of membrane phospholipids.

One of the key features of the HSL is depletion of the metabolic substrates ATP[42] and 2,3-DPG[43]. The energy dependent processes of RBCs are constantly utilizing available ATP stores, when the supple of glucose in the storage medium is exhausted, the RBCs are unable to synthesise ATP and levels begin to fall. As previously mentioned, ATP is necessary for the maintenance of RBC membrane asymmetry; ATP depletion results in loss of membrane asymmetry, most critically exposure of PS on the outer membrane leaflet[19, 20]. The exposure of PS on the outer membrane leaflet causes the RBCs to be recognised and removed from circulation by the reticuloendothelial system[19, 20]. Depletion of ATP also inhibits the activity of membrane cation (Na^+/K^+) pumps, this leads to a loss of osmotic balance as K^+ ions leech out of the cell, and Na^+ ions enter the cell[44]. This loss of osmotic balance is associated with changes in cytoplasmic viscosity and surface area to volume ratio, and loss of cell shape; both of which are associated with a loss of cell deformability[16, 44]. ATP loss is also thought to play at least a partial role in

microvesiculation leading to membrane loss and eventually echinocytosis and spherocytosis[44]. 2,3-DPG is a side product of the Embden-Myerhof pathway of ATP synthesis, produced at the expense of ATP. 2,3-DPG is converted from 1,3-diphosphoglycerate (1,3-DPG), one of the intermediary products of the Embden-Myerhof pathway, through an alternative pathway known as the Luebering-Rapappor shunt[34, 45-47]. Instead of being converted into 3-phosphoglycerate by phosphoglycerate kinase in an ATP generating step, diphosphoglycerate mutase converts 1,3-DPG into 2,3-DPG. The activation of this alternative pathway is regulated by the local conditions; high O_2 concentrations, low 2,3-DPG concentrations and high pH all favour 2,3-DPG production over ATP production[45-47]. 2,3-DPG is a key haemoglobin binding modifier for O_2 that promotes the dissociation of oxygen for release in the tissues; depletion of 2,3-DPG increases haemoglobin's binding affinity for oxygen[48, 49].

Another key factor in the HSL is the accumulation of oxidative stress injury, associated with haemoglobin oxidation and membrane protein oxidation and lipid peroxidation. The RBC cytoplasm is an oxygen rich environment, with a high concentration of proteins, lipids and iron ions. *In vivo* the iron ions are protected from oxidative attack, bound to haemoglobin in their ligand form[50, 51]. Antioxidant molecules such as reduced glutathione, and NADPH produced through the hexose-monophosphate shunt of the Embden-Meyerhof pathway, protect proteins and lipids from oxidation[50, 51]. Under storage conditions, RBCs appear to lose this protection, and haemoglobin auto-oxidation occurs, leading to the accumulation of denatured haemoglobin proteins such as metHb[52]. These denatured haemoglobin proteins are thought to promote membrane lipid and protein oxidation[53, 54]. Oxidative damage in the membrane and cytoskeleton has been associated with echinocytosis and the shedding of microvesicles; the weakened bonds between the membrane and underlying proteins allow the membrane to separate from the cytoskeleton and eventually vesiculate[55].

1.2.2. Measures of *in Vitro* RBC Quality

Much like the manufacturing of other pharmaceuticals, the production of blood components is tightly regulated to ensure patient safety. Components for transfusion must meet standards set by national regulatory agencies. In Canada stored RCC units must meet the standards set by the Canadian Standards Association. As well as adhering to external regulations, blood services also employ internal quality control (QC) systems to further monitor production output. Canadian Blood Services regulations stipulate that at least 1% of production output from each processing site be selected for QC testing[56]. Canadian Blood Services RCC QC testing includes the following parameters: haemolysis (%), unit volume (mL), unit haemoglobin (g), haematocrit (%) and residual white blood cell (WBC) count (#/unit)(table 1.3)[56]. These parameters are implemented to cover a broad view of manufacturing consistency and storage efficiency. Unit volume, haemoglobin content, haematocrit and residual WBC count all represent manufacturing process consistency. Haemolysis is the only QC parameter employed by Canadian Blood Services that represents RBC storage injury.

Haemolysis, the lysis of RBCs, represents the final manifestation of the HSL; the result of the accumulated injuries sustained *ex vivo*. However, as we have seen, there is a broad scope to the pre-haemolytic injury that occurs during storage. Whilst it would not be necessary for blood services to monitor all of the manifestations of the HSL, some may provide opportunities to improve quality. In order to give the most appropriate measure of product quality, it is important to understand the potential clinical implications associated with the HSL.

1.2.3. *In Vivo* Implications of *In Vitro* RBC Quality

The risks associated with blood transfusion have decreased substantially over the years, thanks to a better understanding of haematology and the causes of adverse transfusion reactions. Blood services have adapted their processes to improve patient safety; such as the implementation of donor screening and pathogen testing to mitigate the risk of infection. Despite major advances there are still risks associated with blood transfusions. The ultimate goal of the blood banking industry is to supply hospitals with safe products for transfusions to improve patients' health. The ultimate indicator of product quality must therefore be clinical outcome. It is necessary then, when studying the hypothermic storage lesion, to consider the clinical implications of the manifestations we observe. Recently there has been much debate regarding the association between hypothermic storage of RBCs and adverse transfusion reactions. A number of studies reported adverse clinical outcomes associated with the transfusion of stored blood products; including infection[57], multiple organ failure[58] and mortality[59]. The number of RCC units transfused and the pre-transfusion storage duration of the RCCs were both presented as independent risk factors for adverse transfusion outcomes[57-59]. However, the small scale clinical studies and retrospective analyses lacked the power to definitively link RCC storage duration with adverse transfusion reactions. Two large scale, randomised studies were performed (Red-Cell Storage Duration Study (RECESS)[60] and Age of Blood Evaluation (ABLE)[61]) in an attempt to generate enough solid evidence to support or refute the existing data. Both studies reported that the age of stored RCCs was not associated with significant differences in transfusion outcomes, and that the use of "fresh" (≤10 days storage) RCCs did not result in decreased mortality compared to the use of "standard" (≥21 days storage) RCCs[60, 61]. In fact, the use of "standard" RCCs was associated with decreased risk of certain adverse transfusion reactions compared to "fresh" RCCs. There were a number of key limitations in both the ABLE and RECESS studies. Firstly the average age of cells transfused cells was 6.1 ± 4.9 days for the "fresh" group and 22.0 ± 8.4 for the "standard" group. Whilst these values do represent typical blood banking

storage durations, data on RBCs stored for longer durations would be useful given that the industry is trending towards longer storage durations. Secondly they did not take into account any RCC characteristics other than storage duration. Since these studies were performed, the field has advanced, and we are now studying a wide range of factors other than storage duration that may influence RCC quality and transfusion outcomes (such as WB processing technique and donor factors). In this thesis we will explore a number of sources of variability present within the blood banking industry. Without taking these potentially confounding variables into account, it is impossible to rule out the influence of storage duration on transfusion outcomes, and therefore it is still appropriate to discuss the potential clinical implications of the HSL. A following study reported that when taking into account manufacturing process as well as storage duration, "fresh" WBF RCCs (1-7 days of storage) were associated with a significant increase in in-hospital mortality rates compared to a reference group (BC RCCs stored for 8-35 days)[62]. This study also highlighted the influence of recipient age on transfusion outcome as a potential confounding factor to be taken into account when assessing RCC safety/efficacy.

As previously discussed; haemolysis, the rupture of RBCs, represents the ultimate manifestation of the HSL. The main cytoplasmic constituent of RBCs is haemoglobin, and so when an RBC lyses, all of this haemoglobin is released into the extracellular medium. When a patient receives an RBC transfusion, all of the extracellular haemoglobin and the cellular debris associated with haemolysed cells are also infused. Cell free haemoglobin has been identified as a potent scavenger of nitric oxide (NO)[63, 64], a powerful vasodilator essential in facilitating the flow of RBCs through the small vessels of the microvasculature[65-66]. Large doses of cell free haemoglobin may result in impaired peripheral blood circulation due to excessive NO scavenging.

Depletion of 2,3-DPG occurs by the second week of storage, and increases the affinity of haemoglobin for O_2, limiting its ability to deliver oxygen to tissues *in vivo*[43]. The cells will begin to re-synthesize 2,3-DPG *in vivo* following transfusion, however it can take up to 72 hours post-transfusion to reach normal physiological levels[67]. Infusing an anaemic or otherwise critically ill patient with RBCs that will freely uptake O_2 from the lungs, but not effectively release it in the tissues could potentially exacerbate the patient's condition. Depletion of ATP during storage is thought to have clinical implications in transfused patients, as well as being associated with a number of *in vitro* RCC changes. Depletion of ATP in stored RBCs is associated with reduced 24 hour post-transfusion cell survival[42]. ATP is released by RBCs in response to shear stress, inducing vasodilation in the surrounding vasculature[68, 69], thus facilitating the flow of RBCs. RBCs depleted of ATP during storage may not be capable of inducing this vasomodulation *in vivo*, effecting peripheral blood flow in the transfused patient.

RBCs undergo a number of morphological changes during storage; shape abnormalities, loss of lipid asymmetry, membrane phospholipid depletion, all of which are interlinked, and can affect the *in vivo* survival and functionality of the transfused cells. During storage erythrocytes lose their discoid shape; firstly developing membrane protrusions or spicula, these cells are known as echinocytes, and eventually becoming spherical spiculed cells, or spherechinocytes[70]. This process is initially reversible, with cells able to recover from the echinocyte phase when returned to physiological pH and replenished with nutrients, however spherocytosis is irreversible[71]. The reduced deformability and PS exposure associated with these shape changes prevent the RBC from easily traversing the microvasculature and promotes cellular aggregation and adhesion to the vascular endothelium[20, 70]. This disrupts oxygen delivery to the tissues, and puts strain on the RES due to the large dose of RBCs expressing PS. Lipid loss and membrane asymmetry loss is thought to be associated with the process of RBC microvesiculation[55, 72]; the "blebbing" and

detachment of small membrane bound vesicles containing membrane and cytoplasmic components of the parent cell. The exact mechanisms controlling microvesiculation, and the cause-effect relation between this process and other features of the HSL are not fully understood. However, it is thought that microvesiculation is a natural cellular process that appears to be highly upregulated in stored RBCs[72, 73]. As well as playing a role in the biomechanical manifestations of the HSL, the transfusion of RBC microvesicles has been linked to some of the immunomodulatory and thrombogenic effects sometimes associated with transfusions[73-75]. A recent study demonstrated how transfusing microvesicles from stored RBCs can prime neutrophils and promote neutrophil localisation in the lungs, using a mouse model[75]. Neutrophil activation in the lungs is a key feature of transfusion related acute lung injury (TRALI), one of the leading causes of transfusion related mortality worldwide[75].

1.2.4. What is quality?

Potential sources for variability in stored RCC products can be identified from donation through to transfusion. Some of this variability is accounted for within the scope of QC systems and regulatory standards, and some variability is even necessary; as is the case with donor blood types. However, QC and regulatory systems can't cover the entire scope of variability within stored RCCs and so a certain degree of variability is accepted and uncontrolled. The potential implications on product safety, efficacy and transfusion outcomes due to this uncontrolled variability may not be fully understood.

As discussed, transfusion outcomes are certainly a multifactorial process, with many patient factors to consider on top of the characteristics of the blood being transfused. However, the blood banking industry tends to apply a one size fits all solution to QC standards. Take, for example,

the Canadian Standards Association regulated standard of 75% 24-hour post-transfusion RBC survival. This number, when taken at face value, gives a reasonable measure of the quality of the RBCs being infused into a patient. However, when considering massively transfused patients, who are likely to be in critical condition if they are receiving a large number of units at once, the potential negative effects of transfusion are amplified. For a patient receiving 8 units of RBCs, 25% of each unit acceptably being removed in 24-hours is putting a large strain on the patient's reticuloendothelial system. The potential clinical implications of the unregulated and accepted variability may effect different populations of transfusion patients differently. Further investigating the sources of variability from donation through to transfusion will help define what "type" of RCC would be best suited to an individual patient.

1.3. Hypothesis & Research Objectives

This thesis will be testing the following hypotheses; 1) Storage induced haemolysis in RCCs is dependent on donor sex and age at time of donation as well as component processing method. 2) Storage induced haemolysis in RCCs is a function of residual plasma volume. 3) RBC size, density and haemoglobin content are dependent on donor sex and age.

The primary research objectives of this thesis are as follows:

Research Objective 1: Using retrospective QC data analysis, identify a relationship between donor factors (donor sex and age at time of donation), component separation processing (WBF vs BC) and post-storage RCC characteristics (unit volume, haematocrit, haemoglobin content and unit haemolysis).

Investigating the pre-storage factors effecting post-storage RCC quality characteristics, with the aim of expanding upon the current understanding of RBC storage injury and the variables that influence it. Merging existing blood bank QC data with donor information and component processing information will allow for detailed group analysis, comparing the *in vitro* quality characteristics of sub-populations of RCCs.

Research Objective 2: Using experimental evidence, and retrospective QC data analysis; identify a correlation between residual storage plasma volume and storage induced haemolysis in RCCs.

Using existing QC data, residual plasma volume will be calculated for Canadian Blood Services RCCs, and its influence on storage haemolysis determined using a statistical model. The effect of residual plasma volume and storage additive solution volume will then be determined experimentally. The study will involve storing RBCs in known volumes of plasma and additive solution and monitoring haemolysis throughout a 42-day storage period (@4°C ± 2°C).

Research Objective 3: Using experimental evidence, and retrospective QC data analysis; demonstrate a correlation between donor factors (age and sex), and the size, density and haemoglobin content of pre-storage and post-storage RBCs.

Further investigating the influence of donor sex and age at time of donation, focussing on morphological differences in RBCs between different donor groups before, during and after a 42-day storage period (@4°C ± 2°C). An assay will be developed in order to quantify cell density, involving centrifugal fractionation of RBCs within a known density gradient.

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1.5. Tables

Table 1.1: Composition of current generation RBC storage additive solutions

	Additive Solution			
Constituent (mM)	**SAGM**	**AS-1**	**AS-3**	**AS-5**
Adenine	1.25	2	2	2.2
Glucose	45	111	55	45
Mannitol	30	41	-	45.5
NaCl	150	154	70	150

Adenine, glucose (dextrose), mannitol and sodium chloride content of the four major licensed red cell additive solutions, in mM.

Chapter 2

Assessing the influence of component processing and donor characteristics on quality of red cell concentrates using quality control data[i]

2.1. Introduction

The collection, processing and storage of transfusable blood products are highly regulated to ensure product quality and patient safety. Product specifications are set by government standards agencies and are reflected in the quality assurance programs of blood services. Blood Services utilise QC systems to monitor production output against these regulatory standards. Despite stringent control mechanisms, a great deal of variability still exists within the blood components transfused [1-3]; a comprehensive understanding of the products being administered is vital to ensure benefit to the patient.

[i] A version of this chapter has been published under this title in Vox Sanguinis, 2016, vol. 111, pg. 8-15.

Factors affecting the characteristics of RCC products can be due to inherent variability in the blood donors, or in the manufacturing processes used by blood services. Many of these variables have been shown to influence product quality [1, 4, 5]; however, the scope of variability within blood components and quality has not been fully explored. While controlling some of these variables may not be necessary or even plausible, investigating their impact on product quality deserves our focus if we are to ensure greater consistency in the products provided to patients.

In an attempt to better understand the relationship between manufacturing, donor and product quality, we assessed Canadian Blood Services' manufacturing and donor records against QC data. Here we present how this kind of QC data analysis can be useful in highlighting sources of variation in product characteristics, and in addressing basic scientific questions about blood components and manufacturing processes. We also discuss the concept of quality within a blood manufacturing environment, speculating as to how clinically significant QC parameters are in practice, and how well current QC systems correlate with transfusion outcomes.

2.2. Materials and Methods

2.2.1. RBC Collection & Processing

Between June 2011 and October 2013, a total of 20,676 RCC units were selected for expiry QC testing, and 21,261 units were selected for residual WBC segment testing. A total of 41,979 unique donations from 14 participating Canadian Blood Services production sites were entered into the QC database. Using unique donation ID's, the QC database was merged with manufacturing

records and non-identifiable donor information: sex, age at time of donation and ABO/RhD blood group. The data was compiled using Excel spreadsheet software (Microsoft, Redmond, WA).[i]

WB donations from healthy, eligible donors were collected into citrate-dextrose-phosphate (CPD) anticoagulant and processed by either the BC [6] or WBF methods [7], according to Canadian Blood Services standard operating procedures as previously described [8, 9] (Figure 2.1). Briefly; WBF units were leukoreduced by passing the WB through a filter; either promptly after collection at room temperature, or within 72 h of collection if stored and filtered at 1-6°C. The LR-WB was then centrifuged at 4552 x *g* for 6 minutes to produce one unit of RCC and a unit of plasma. Processing was completed within 8 h of the stop bleed time, or within 72 h if filtered at 1-6°C. BC units were centrifuged prior to leukoreduction at 3493 x *g* for 11 minutes, yielding a unit of buffy coat in addition to the plasma and RCC. Separated BC-RBCs were leukoreduced at room temperature within 24 hours of stop bleed time. Separated RCC units were stored in SAGM additive solution at 4±2°C for a maximum of 42 days.

2.2.2. Quality Control (QC) Testing

Canadian Standards Association stipulates that at least 1% of manufactured product at each site must be selected for QC testing every month in order to monitor production; these units are sent to one of two QC testing labs. Testing was performed at product expiry (day 43 after processing), and the units were destroyed following testing.

[i] WB collection, component processing and QC testing was performed by Canadian Blood Services staff. Construction of the merged database was performed by myself.

Before any sampling was performed, unit volume was calculated by weighing the unit and dividing the mass by a standardised density figure (1.06 g/mL). Total haemoglobin (Hb) was measured using an Advia 120 Hematology System (Siemens AG, Erlangen, Germany); RBCs are mixed with Advia 120 HGB reagent (Siemens AG; 20 mmol/L potassium cyanide, 2.0% dimethyllaurylamine oxide) which results in haemolysis, and reacts with the exposed haem-iron causing oxidisation and cyanisation. The reaction product is measured colorimetrically at 546 nm and is directly proportional to total haemoglobin content. Unit haematocrit (Hct) was also determined using an Advia 120 Haemotology System; RBCs were treated with Advia 120 RBC/PLT reagent (Siemens AG; sodium dodecyl sulfate, 0.035 mmol/L, disodium EDTA dehydrate, 4.03 mmol/L, tetrasodium EDTA dehydrate, 3.36 mmol/L, sodium chloride, 109.3 mmol/L, glutaraldehyde 0.11% w/v), which causes them to lose their biconcave shape and become spherical, and acts as a fixative. The number of cells and mean cell volume (MCV) are then measured using flow cytometry. Unit Hct is calculated using the formula Hct = (RBC count x MCV)/10.

Supernatant haemoglobin was measured using a Plasma/Low Hb Photometer (HemoCue, Ängelholm, Sweden); supernatant samples were loaded into microcuvettes pre-coated with assay reagents (Plasma/Low Hb Microcuvettes, HemoCue), supernatant haemoglobin is converted to ferric metheamoglobin by sodium nitrate, which then reacts with sodium azide to form azidemethaemoglobin, which is measured photometrically at 570 nm and 880 nm. Haemaglobin concentration is calculated from a standard curve against tri-level haemoglobin controls (R&D Plasma Hemoglobin Hematology Controls, R&D Systems, Minneapolis, MN). Percent haemolysis (Hly) was calculated using the formula Hly = [supernatant Hb x (1 – Hct)]/total Hb [9].

Residual WBC testing was performed within 24 hours of processing as WBCs will break down during storage; therefore, this data is from a different cohort of units to the rest of the QC data. Samples for residual WBC testing were prepared from sealed segment lines and shipped to a QC testing facility (Calgary, AB & Ottawa, ON), where WBC count was performed using flow cytometry, as previously described [10].

2.2.3. Assessing additive solution delivery in WBF and BC collection sets

As a potential factor in the association between WB processing method and RCC storage haemolysis, the delivery of SAGM additive solution was assessed in collection sets used by Canadian Blood Services.[i] The volume of SAGM in Fenwal and Macopharma WBF and BC sets was assessed using "empty" bags, not containing RBCs. For WBF sets, collection bags and tubes were removed using a clamp sealer, leaving the RBC storage bag and the SAGM bag. The connecting tube between the storage bag and SAGM container was cut and secured into a measuring cylinder, the cannula on the SAGM container was then broken, and the SAGM allowed to drain into the cylinder, and the collected volume was recorded. For BC sets, collection bags and tubes were removed using a clamp sealer, leaving the SAGM/RBC storage bag, the satellite bag, and the connecting tubing and leukoreduction filter. The SAGM was allowed to drain into the satellite bag, the tube was then clamped and cut. The open tube was secured into a measuring cylinder, the clamp was removed and the SAGM was allowed to drain through the filter into the cylinder. The collected volume was recorded.

[i] Dr. Jason Acker and I were responsible for designing the experimental method used to assess additive solution volume in processed RCCs. Data collection was performed by myself.

2.2.4. Statistical analysis

Statistical analysis was completed using statistical analysis software (SAS 9.1, SAS, Cary, NC. and SPSS 21, IBM, Armonk, NY). The distribution of each parameter was assessed for normality using measures for skewness and kurtosis; mean and standard deviation (SD) were compared for normally distributed parameters, whilst median and range were compared as well as mean and SD for non-normally distributed parameters. Mixed model analysis of variance was performed to compare mean QC parameters grouped based on donor factors and processing conditions. For each outcome variable, a univariate mixed model with a single predictor was first fitted, other variables were then added to the model to assess the adjusted effect of the initial predictor after potential confounding from the each added variable. A p-value of less than 0.0001 was considered significant.[i]

The presence of repeat donors within the QC database has a potential clustering effect on the data. To account for this, a random effects model was applied, using unique donor ID's as the random effects variable. Due to the presence of this repeat donor population, specifically those that had at least one donation processed using each method chosen for QC testing, it was also possible to assess the influence of component processing conditions on RCC characteristics within a single donor. This removes additional confounding variation between donors that is not covered within the scope of the database, and could therefore not be fitted to the model.

[i] Qi Long Yi performed the mixed model analysis of the database according to test parameters conceived by Dr. Jason Acker and I.

2.3. Results

2.3.1. Manufacturing processes affect RCC QC parameters

The database parameters associated with manufacturing conditions are processing method (WBF & BC), storage bag manufacturer, and processing site. Units manufactured using the BC process had smaller unit volumes ($p < 0.0001$) and therefore lower total unit hemoglobin values ($p < 0.0001$) compared to WBF units (Table 2.1). BC units also had lower average post-storage hemolysis values ($p < 0.0001$); however, both processing methods lie well below the regulated standard of 0.8% (Table 2.1). Bag manufacturer also had a significant influence on post storage hemolysis ($p < 0.0001$). Units produced in Fenwal collection sets had significantly lower residual WBC counts than those produced in Macopharma bags ($p < 0.0001$). Processing method affected residual WBC count to a lesser extent, though still significant ($p<0.0001$; Table 2.2). Accurate analysis of site-to-site differences is difficult to perform, as there are many un-reported variables associated with the manual stages of processing.

2.3.2. Manufacturing processes affect RCC storage medium composition

Using the standard QC data, it is possible to extrapolate additional information regarding the composition of the manufactured units. Supernatant volume (assumed to be the volume of additive solution, plus any residual non-cellular content following component separation) was calculated using the formula:

$$Supernatant = \frac{Volume \times (1 - (Hct))}{100}$$

BC processed units had lower average supernatant volumes than WBF processed products (Table 2.2). To calculate the residual non-cellular volume (labelled "residual plasma") the average recorded volume of SAGM additive solution was subtracted from the calculated supernatant volume. Larger residual plasma volumes were calculated for BC products compared to WBF products ($p < 0.0001$). Other manufacturing and donor factors did not have a significant effect on the supernatant or residual plasma volumes.

2.3.3. Donor characteristics affect RCC hemoglobin and storage hemolysis

Both donor sex and age at time of donation had an effect on storage quality. Units from male donors exhibited higher hemolysis than units from females (Figures 2.2 & 2.3, $p < 0.0001$), while increasing donor age also correlated with increasing hemolysis ($p < 0.0001$; Figure 2.3). Units from female donors had lower hematocrit and total hemoglobin (64% Hct, 53.2 g/unit Hb) than their male counterparts (67% Hct, 61.2 g/unit Hb, $p < 0.0001$), whereas donor age had no effect on these parameters.

2.3.4. Effect of QC selection on database profile

While all QC testing (apart from residual WBC count) was performed at expiry (43 days), the average age of Canadian Blood Services products when they are issued to hospitals ranged from 7-17 days, and was dependent on donor ABO/Rh group (Table 2.3). The representation of different ABO/Rh groups in the QC database was inverse to that in the donor population; where B + and AB+ dominate the QC database, while A+ and O+ are the most abundant donor groups (Table 2.3).

Within the database there is a population of donors that have been selected for QC testing multiple times, and repeatedly exhibit high hemolysis (Table 2.4). The average post-storage hemolysis of units from this population is greatly increased compared to the remaining population. Comparing hemolysis values for repeat donors that have donated in both WBF and BC sets (684 donors) allows us to account for the effect of donor variation on hemolysis. In this group of donors, WBF units displayed significantly higher hemolysis than BC processed units (WBF: 0.39 (± 0.26), BC: 0.23 (± 0.11), mean Δ: 0.16 (± 0.21); $p < 0.0001$).

2.4. Discussion

In this study we show that in depth analysis of QC data can be used to highlight, and better understand sources of variability within RCC products. We combined QC data with manufacturing records and donor information in order to understand the relationship between donor characteristics, manufacturing processes, and the quality of stored RCC products. We observed increased storage hemolysis, total Hb and unit volume in WBF processed RCCs compared to BC products, while BC processed units were found to contain greater volumes of residual plasma than WBF products. RCCs processed in Fenwal collection sets were found to contain fewer residual WBCs than their Macopharma counterparts. We also observed a donor influence on RBC storage injury; units from male donors exhibited greater storage hemolysis than those from female donors, with hemolysis also increasing with donor age at time of donation.

Hemolysis during RCC storage is a manifestation of the hypothermic storage lesion - the damage that RBCs sustain under storage conditions, commonly associated with extended storage periods [11, 12]. Hemolysis represents the release of hemoglobin into the extracellular fluid through either RBC lysis or the shedding of microvesicles containing Hb [13]. Hb is a potent scavenger of NO [14,

15], which is released from RBCs during oxidative and shear stress, and acts as a vasodilator [16, 17]. Transfusing an already compromised patient with a large dose of free Hb could have adverse vasoregulatory implications [18-20]. The greater storage hemolysis observed in WBF processed units compared to BC units is a manifestation of the increased damage accrued throughout processing and storage.

The key differences between the WBF and BC processing methodology are the point of filtration, and the component separation process. WBF processing exposes the WB to greater centrifugal force which could have a more mechanically damaging effect on the cells [21], as well as concentrating free and microvesicle bound extracellular Hb into the RCC component. It is also possible that the WBF process gives rise to a less favourable RBC storage environment than the BC process.

A number of compositional differences between BC and WBF units were identified by examining the QC and manufacturing data which could influence storage hemolysis. One of the biggest differences seen is that of unit volume, with WBF units being substantially larger than BC units on average. This disparity is to be expected with the removal of the "buffy coat" component in BC processing [6]. Supernatant volumes across all products averaged 103 mL, less than the volume of SAGM assumed to be added before storage. To further investigate this issue, the effective delivery of SAGM to the RCC storage container was assessed in different collection sets. The substantial loss of SAGM volume in BC sets is likely attributed to the requirement of BC filters to be "pre-wet" with additive solution prior to leukoreduction. Previous studies into the effect of additive solution volume on RBC storage have found increased volume of additive solution to be associated with reduced hemolysis, and decreased RBC ATP loss during storage [22]. The impact

of the residual non-cellular, non-additive solution component; labelled “residual plasma” has not been fully elucidated [24]. Plasma has been shown to promote RBC survival in vitro, suppressing phosphatidylserine expression and programmed cell death [25]. The immunomodulatory effects of RBC transfusion have been attributed to residual WBCs and WBC derived cell debris, and human leukocyte antigen (HLA) peptides in donor plasma [29]. As the volume of plasma was shown to be dependent on manufacturing method, whereas pre-storage WBC count varied base on bag manufacturer, these factors should be considered in studies examining transfusion related immunomodulation and TRALI.

In addition to the manufacturing conditions affecting product quality, we also assessed the influence of donor related factors. Fresh frozen plasma from female donors has been almost universally removed from blood bank inventories, due to the proposed increased risk of TRALI [30]. As the increased hemolysis values we observed in male donors are consistent with previous studies [31], it is possible that compositional differences between male and female blood make for more/less favourable RBC storage conditions [31, 32]. The age of the donor at the time of donation also affected product quality, donations from older donors showed increased hemolysis compared to younger donors. These observations demonstrate how donor factors can influence storage and quality, however the scope of variability in the donor population is far broader than the parameters included in the database [13, 14].

Linking donor information to the QC data allowed us to identify a population of QC units from repeat donors within our dataset. Within this population we identified a sub-group of repeat donors who have donated more than one unit exhibiting high hemolysis (>0.65%). These high hemolysis donors could represent poor storage phenotypes, or underlying asymptomatic RBC

pathologies, and warrant further identification and investigation of this sub-group. Donor screening is already an essential practice for blood services, with a better understanding of how donor factors influence storage, donations could be tailored to more optimal storage systems, and poor storing donors removed from the system. The existence of a repeat donor population also provided a means of accounting for donor variability when assessing manufacturing conditions. Identifying a small population of donors that had donated in both BC and WBF collection sets, we were able to show that the increased hemolysis observed in WBF processing was not due to confounding donor factors.

Some of the key limitations of this study reflect the limitations inherent in the QC program from which we collected our data. We have discussed the effects of variability on product quality in the context of QC parameters; however, the ultimate measure of product quality must be patient outcome. The clinical significance of the observations presented here can only be speculated as clinical outcome data is not available, nor could it be collected for the units selected for destructive QC testing. With this being the case, how representative is the QC data of the overall donor/unit population? A destructive QC selection process is inherently biased, in that it is entirely based on the products available at expiry, which in turn is dependent on demand. This is most obvious in the ABO/Rh group bias, where common blood groups are under-represented in the QC database as these products are in constant demand and issued to hospitals long before expiry. The age of all units at the time of QC testing was 43 days (within 24 hours of product expiry), however the average storage duration of transfused products is much shorter. In addition, storage related injury will be less severe in most transfused products compared to the extended storage duration represented in the QC data. In addition to the inherent limitations due to QC selection bias, statistical analysis of databases of this scale and complexity also introduces some limitations.

With such large sample sizes, we encountered extremely low p-values in our analyses; in order to try and mitigate this effect we used much more stringent than usual thresholds of significance.

Combining the compiled databases with patient outcome information would provide context to the variations observed in this study, and glean insight into how effectively current QC systems align with actual patient safety/treatment efficacy. In this study we analysed QC data from Canadian Blood Services only, focusing on RCC products, thus only covering a portion of blood components manufactured in Canada. Expanding this analysis across multiple national QC programs would provide a much greater opportunity to look at the influence of processing variables and donor demographics. The limitation here is that blood services collect QC, donor and manufacturing data to varying degrees, hampering the kind of in-depth of analysis that was presented in this study. An international effort to collect comprehensive production, donor and clinically significant quality data might be the necessary course of action, allowing for large scale benchmarking and allowing us to tailor more effective donor/recipient oriented QC systems.

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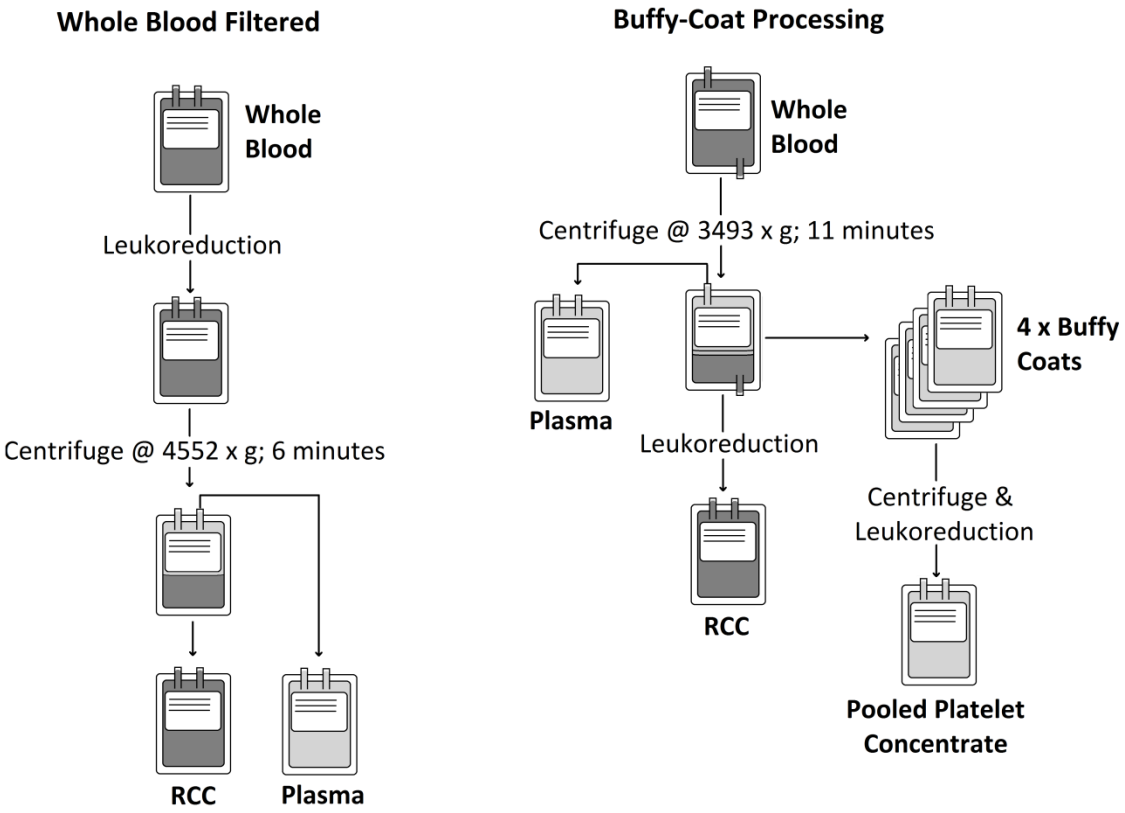
2.6. Figures and Tables

Figure 2.1: RCC manufacturing processes at Canadian Blood Services



Schematic representing the WBF and BC whole blood processing methods used by Canadian Blood Services. Following component separation, RBC component is mixed with SAGM additive solution and stored at 4±2°C. WBF hard spin: 4552 x *g* for 6 minutes. BC hard spin: 3493 x *g* for 11 minutes.

Table 2.1: The effect of manufacturing conditions on product characteristics

	Volume (mL)	**Hct** (%)	**Total Hb** (g/unit)	**Supernatant Hb** (mg/dL)	**Hemolysis** (%)
BC (n = 10,928)	279 (±17.6)*	65 (±3.0)	53.0 (±6.1)*	125 (±83)	0.23 (±0.15)*
Fenwal (n = 7,092)	278 (±17.2)	65 (±3.0)	52.7 (±5.8)	118 (±83)	0.22 (±0.16)†
Macopharma (n = 3,836)	281 (±18.1)	65 (±3.0)	53.2 (±6.5)	138 (±80)	0.25 (±0.13)†
WBF (n = 9,748)	330 (±18.5)*	66 (±3.0)	63.2 (±6.1)*	211 (±142)	0.37 (±0.25)*
Fenwal (n = 7,287)	332 (±18.0)	66 (±3.0)	63.7 (±6.0)	212 (±148)	0.38 (±0.27)†
Macopharma (n = 2,461)	322 (±17.7)	67 (±3.0)	62.7 (±6.3)	208 (±122)	0.35 (±0.20)†
Total (n = 20,676)	303 (±30.9)	66 (±3.0)	58.1 (±6.1)	165 (±122)	0.29 (±0.22)

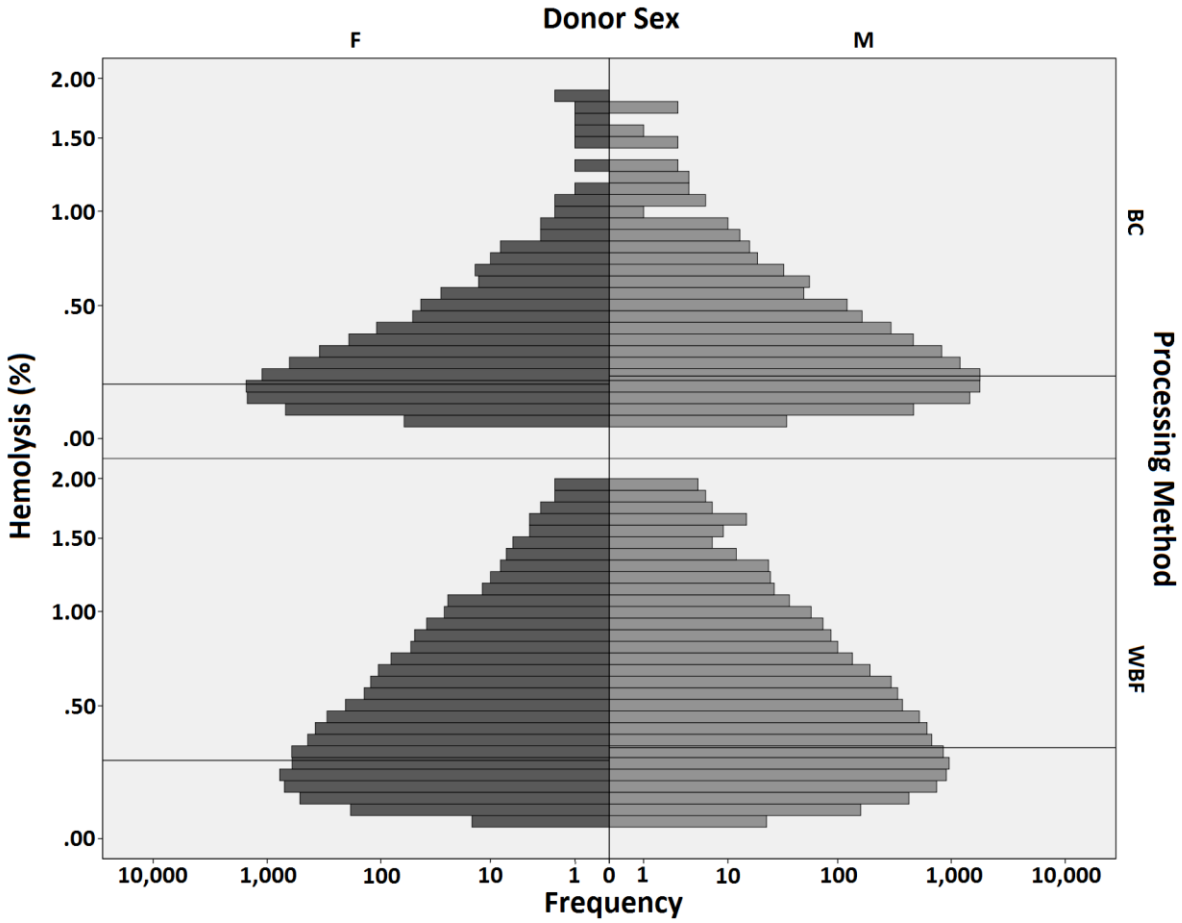
Data from units tested within 24 hours of product expiry (42 days of storage). Mean values ± 1 SD. * indicates significant difference ($p < 0.0001$) between BC and WBF manufacturing methods. † indicates significant difference ($p < 0.0001$) between bag manufacturer alone based on univariate analysis; not significant when adjusted for processing method.

Table 2.2: The effect of manufacturing condition on unit composition

	***SAGM Vol** (mL)	**Supernatant Vol** (mL)	**Residual Plasma Vol** (mL)	**Plasma : SAGM ratio**	**Residual WBC count**
BC	75.1 (±3.6)	97 (±6.6)*	20.9 (±7.4)*	0.27 (±0.11)*	0.25 (±3.41)*
Fenwal	78.4 (±1.6)	97 (±5.8)	18.4 (±6.1)	0.24 (±0.08)	0.01 (±0.16)†
Macopharma	71.8 (±0.8)	97 (±7.4)	26.3 (±8.7)	0.37 (±0.10)	0.57 (±5.76)†
WBF	103 (±2.7)	109 (±9.2)*	8.47 (±8.5)*	0.08 (±0.08)*	0.31 (±3.43)*
Fenwal	106 (±0.9)	113 (±9.8)	8.87 (±8.7)	0.08 (±0.08)	0.16 (±0.28)†
Macopharma	101 (±1.1)	105 (±8.6)	7.03 (±7.9)	0.07 (±0.08)	0.77 (±6.80)†

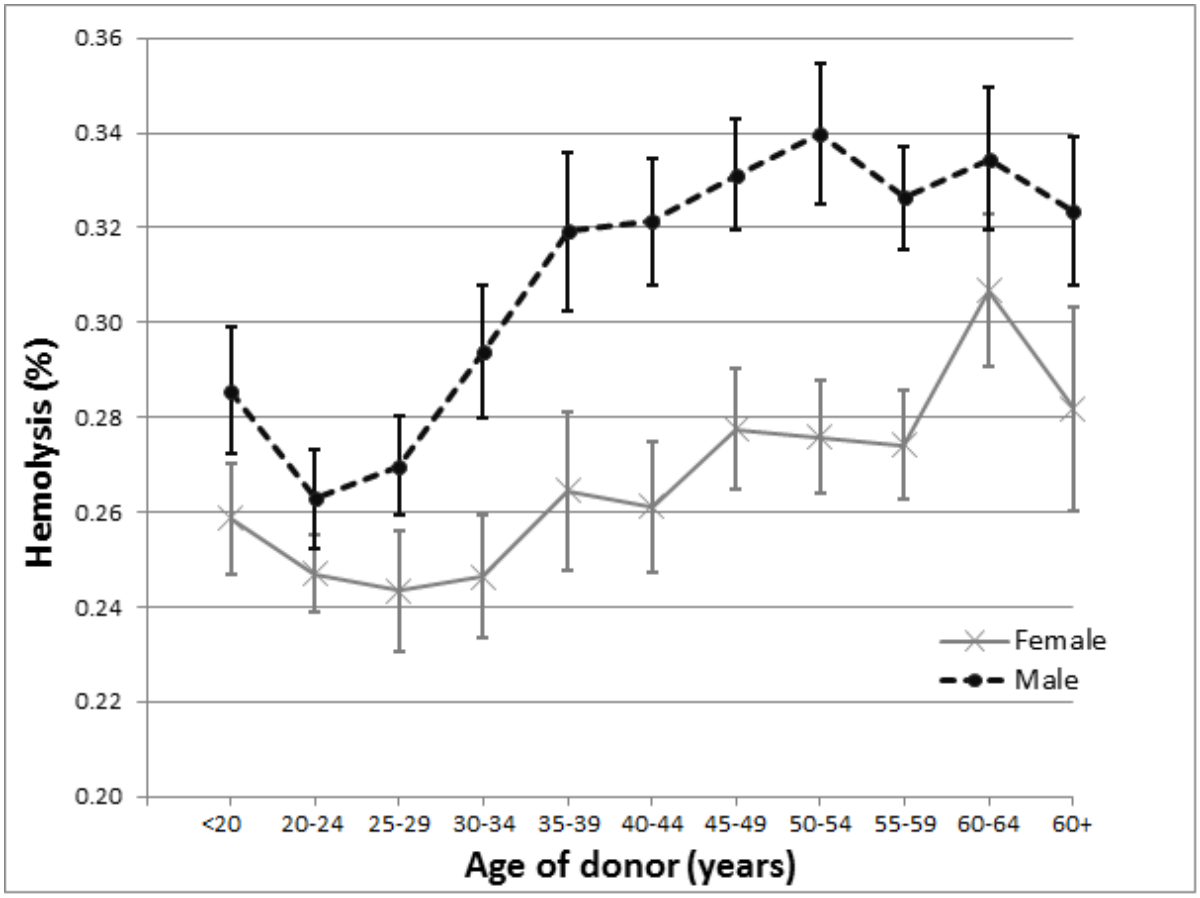
Mean values ± 1 SD. * Average volume of SAGM delivered to the RBC storage container determined experimentally using "dry" collection sets not containing RBCs (n = 40). Supernatant volume, residual plasma volume and SAGM : Plasma ratio calculated from QC/experimental data (n = 20,676). Residual WBC testing was performed separately to the other QC parameters, total residual WBC *n* = 21,261. *indicates significant difference ($p < 0.0001$) between BC and WBF manufacturing methods. †indicates significant difference ($p < 0.0001$) between bag manufacturer.

Figure 2.2: Donor sex and processing method influence RCC storage haemolysis



Frequency histogram showing storage hemolysis distribution for female vs. male donors, and BC vs. WBF processing. Data from units tested within 24 hours of product expiry (42 days of storage), n = 28,227. Reference line represents median hemolysis for each group (BC F = 0.18, BC M = 0.21, WBF F = 0.27, WBF M = 0.32).

Figure 2.3: Donor sex and age influence RCC storage haemolysis



Data from units tested within 24 hours of product expiry (42 days of storage). Mean haemolysis values for female (solid line) vs. male (dashed line) donors based on donor age at time of donation. Error bars represent 95% CI, n = 20,676. Units from male donors exhibit higher post-storage haemolysis ($p < 0.0001$). Increasing age at time of donation correlates with increased post-storage haemolysis.

Table 2.3: Blood group distribution and storage duration in QC database vs. donor population

	QC Database		**Canadian Blood Services Donor Population**	
Blood Group	**Frequency** (% of Total)	**Storage Duration** (Days)	**Frequency** (% of Total)	**Storage Duration** (Days)
A+	6.8	42	36	9
A-	0.1	42	6.0	8
B+	60	42	7.6	11
B-	0.3	42	1.4	8
AB+	29	42	2.5	17
AB-	0.2	42	0.5	11
O+	2.3	42	39	7
O-	0.1	42	7.0	7

Distribution of blood groups (ABO/Rh) within QC database and Canadian Blood Services donor population, and average storage duration (until distributed to hospitals) for blood groups within these two populations. Donor population based on Canadian population data and Canadian Blood Services manufacturing records. QC database does not accurately represent the population of transfused products.

Table 2.4: Repeat donor populations

	QC database	Repeat donors*	High haemolysis donors†
Number of units	20,676	6,808	230
Mean number of donations per donor	1.23	2.36	1.41
Haemolysis (%)	0.29 (±0.22)	0.30 (±0.23)	0.94 (±0.41)
% of donations with haemolysis > 0.65%	5.2%	5.6%	86.5%
% of donations with haemolysis > 0.80%	2.8%	2.9%	54.3%

* Repeat donors refer to those who have had donations selected for QC testing more than once.

†High haemolysis donors represent repeat donors who have had more than one donation selected for QC testing exhibiting a haemolysis value higher than 0.65%, the upper limit of the 90% CI for haemolysis in the database.

Chapter 3

Supernatant Composition of Stored RCC Components Influences the Rate of Storage Associated Haemolysis

3.1. Introduction

During WB component processing, donor RBCs are separated from the plasma and platelets through fractionation in a centrifuge. Using currently available technology, it is not possible to achieve complete separation of the components, meaning a small amount of residual plasma remains with the RBC component during storage. The presence of residual plasma, and its influence on transfusion outcomes has been studied mostly due to the association between plasma components and transfusion related injury and immunomodulation. Most notably; transfusion components associated with plasma (e.g. HLAs[1, 2], human neutrophil antigens (HNAs)[3] and lysophosphatidylcholines (lysoPCs)[4]) have been shown to contribute to the development of TRALI, with greater risk of TRALI being associated with larger volumes of residual plasma[4, 5].

More recently, however, the influence of residual plasma on the *in vitro* characteristics of stored RCCs has come into question. A recent study by Bashir *et al.* investigating neonatal RCC quality (which are purposely stored in a mixture of donor plasma and additive solution), reported increased storage haemolysis in neonatal RCCs stored in lipaemic plasma compared to non-lipaemic plasma[6]. De Korte *et al.* then observed this phenomenon in adult RCCs stored with different quantities of lipaemic and non-lipaemic residual plasma. The triglyceride content of the plasma was found to have a much more significant influence on storage haemolysis than the total volume of residual plasma[7].

Previously, we demonstrated that WB processing conditions influence both the quality and composition of stored RCCs[8]. One notable compositional difference we observed between WBF and BC processed units was the ratio of residual plasma to additive solution. Our initial calculations based on Canadian Blood Services QC data suggested an increased ratio of plasma to additive solution in BC processed units compared to WBF processed units. When comparing multiple methods for calculating the volume of residual plasma in RCCs, we observed a great amount of variability between calculations. Although our measurements were inconclusive; the observed variability in residual plasma volume and additive solution volume based on processed method may be related to the associated quality differences in RCCs between processing methods.

In this investigation, we set out to determine the influence of residual plasma to additive solution ratio on the quality of stored RCC components. Using a range of plasma : SAGM ratios, including the ratios we observe in WBF and BC processing, we aim to determine if these compositional changes have an effect on RCC haemolysis levels and other *in vitro* quality parameters throughout storage.

3.2. Materials and Methods

The aim of this study was to quantify any potential influence residual plasma and additive solution volume has on RCC storage haemolysis. Due to the observations made previously suggesting donor sex and method of component separation have a significant influence on RCC haemolysis during storage[8], a stratified study design was chosen. The four resulting sample groups for the study were: Male donor WBF processed, male donor BC processed, female donor WBF processed and female donor BC processed. This study design was intended to mitigate the confounding effects of donor sex and component separation method on the level of storage haemolysis.

3.2.1. Whole blood collection and processing

WB was collected from 3 ABO/Rh compatible male donors and 3 ABO/Rh compatible female donors using Macopharma WBF collection sets (DQE 7291 LX Leucoflex MTL1 Quadruple Top and Top System, CPD/SAGM 500 mL, Macopharma, Tourcoing, France), and from 3 ABO/Rh compatible male donors and 3 ABO/Rh compatible female donors using Macopharma BC collection sets (LQT 7291 LX leucoflex LCR-Diamond Quadruple Bottom and Top System, CPD/SAGM 500 mL), and processed according to Canadian Blood Services standard operating procedures, as previously described (Chapter 2 – 2.2.1, Figure 2.1)[9][i].

3.2.2. Storage series sample preparation

In order to assess the influence of residual plasma and additive solution on storage haemolysis, it was necessary to account for confounding from the other factors affecting storage haemolysis,

[i] WB collection and component processing performed by netCAD staff at the Centre for Blood Research, University of British Columbia, Vancouver, BC.

such as those discussed in chapter 3. Processed RCCs were pooled based on donor sex and processing method; 3 x female WBF, 3 x male WBF, 3 x female BC, 3 x male BC. To pool a group of units, each unit in the pool was docked to a large volume container (1000 mL Component Bag, Haemonetics Corporation, Braintree, MA, USA) using a sterile docking device (Compodock, Fresenius HemoCare, Bad Homburg, Germany) and the contents transferred one at a time, the pool is then mixed gently by inversion. From each pooled group, 3 replicate storage series were prepared[i]:

Female WBF: 1-A, 1-B, 1-C	Male WBF: 2-A, 2-B, 2-C
Female BC: 3-A, 3-B, 3-C	Male BC: 4-A, 4-B, 4-C

To prepare the storage series, 11 x 20 mL samples were transferred from the pooled unit into labelled 50 mL plastic conical tubes (Falcon 50mL High Clarity PP Centrifuge Tube, Corning Incorporated, Corning, NY, USA) for washing. RBC samples were spun at 2200 x g for 10 minutes at 4°C (5810 R Centrifuge, Eppendorf, Hamburg, Germany) and the supernatant was aspirated and discarded, 0.9% w/v sodium chloride (NaCl) was added to bring the sample volume back up to 20 mL. Samples were mixed thoroughly by inversion before being spun again at 2200 x g for 10 minutes at 4°C. Following the second centrifugation step, the supernatant was again aspirated and discarded; 12 mL of each remaining concentrated RBC sample was transferred into fresh 50 mL falcon tubes labelled 1-11. Concentrated RBC samples were re-suspended in a mixture of SAGM and plasma (autologous to one of the donors in the pool) according to table 1, and mixed

[i] Storage series sample preparation and albumin concentration validation was performed by myself. I would like to acknowledge Tracey Turner for providing training and supervision in the necessary protocols.

thoroughly by inversion. Pooling and sample preparation was performed at room temperature within 72 h of stop bleed time.

3.2.3. Validation of storage series

Following sample preparation, the albumin concentration in each sample was measured using a detection assay (BCG Albumin Assay Kit, Sigma Aldrich, St. Louis, MO, USA) to demonstrate that the plasma : SAGM ratio of each sample was as defined by the target series. 700 µL of re-suspended RBCs were transferred from each sample into a 1.5 mL microtube and centrifuged at 2200 x g for 10 minutes. 10 µL of supernatant was aspirated from the centrifuged samples and diluted two-fold with water. Diluted supernatant samples were mixed with bromocresol green reagent mixture (BCG Albumin Assay Reagent, MAK124A, Sigma Aldrich), which binds selectively to albumin to form a green compound, the absorbance of which was determined spectrophotometrically at 620 nm (SpectraMax 384+, Molecular Devices, Sunnyvale, CA, USA). Albumin concentration was then calculated from a standard curve of known albumin concentrations (BCG Albumin Assay Albumin Standard, 5 g/dL, MAK124B, Sigma Aldrich).

3.2.4. Sample storage and *in vitro* RBC quality testing

Prior to storage each sample was assessed for haemolysis, mean cell volume (MCV) and mean cell haemoglobin (MCH)[i]. Haematocrit was measured visually by centrifuging a sample in a capillary tube for 5 minutes at 14,850 x g (Haematokrit 2010, Andreas Hettich GmbH, Tuttlingen, Germany). To determine total haemoglobin, 5 µL of each sample was diluted in Drabkin's reagent [1:200] (0.61 mmol/L potassium ferricyanide, 0.77 mmol/L potassium cyanide, 1.03 mmol/L

[i] *In vitro* RBC testing throughout storage was performed by myself. I would like to acknowledge Tracey Turner for providing training and supervision in the necessary protocols.

potassium dihydrogen phosphate, 0.1% vol/vol Triton X-100. Sigma Aldrich) which causes the RBCs to lyse, and converts the released haemoglobin into methaemoglobin, then cyanmethaemoglobin. 200 µL of each sample was loaded into a 96 well plate, and the concentration of cyanmethaemoglobin was measured spectrophotometrically at 540 nm (SpectraMax 384+, Molecular Devices), haemoglobin concentration was calculated against a standard curve of known haemoglobin controls (Trilevel Hb Controls, StanBio Laboratory, Boerne, TX, USA). To determine supernatant haemoglobin, samples were first centrifuged at 2200 x g for 10 minutes at 4°C, 20 µL of supernatant was removed and diluted in Drabkin's reagent [1:25] and haemoglobin values were again read at 540 nm. Haemolysis was calculated using haematocrit, total haemoglobin and supernatant haemoglobin concentrations, using the formula Hly = [supernatant Hb x (1 – Hct)]/total Hb. MCV and MCH were determined using a Coulter haematology analyser (Coulter AcT 8 hematology analyzer, Beckman Coulter Inc., Fullterton, CA, USA).

Samples were stored in 50 mL falcon tubes at 1-6°C for 42 days. Haemolysis, MCV and MCH assessments were repeated on stored samples at days 21 and 42 of storage, samples were mixed thoroughly by inversion prior to testing.

3.2.5. Statistical Analysis

Statistical analysis was completed using statistical analysis software (SAS 9.1, SAS, Cary, NC. and SPSS 21, IBM, Armonk, NY)[i]. Regression analysis was used to quantify the effect of increasing plasma concentration on *in vitro* RCC characteristics for each series, R^2 values were calculated and p-value of $p < 0.05$ was considered significant. The distribution of haemolysis was assessed

[i] Statistical analysis was performed by Qi Long Yi and I. I would like to acknowledge Qi Long Yi for guidance and help choosing and performing the appropriate statistical tests, and help writing the Statistical Analysis section of this chapter.

for normality using measures for skewness and kurtosis; the data was found to be not-normally distributed, therefore median and range were compared as well as mean and SD. Haemolysis values for each pooled donor group were compared using a non-parametric Pearson's chi-squared test, and p-value of $p < 0.05$ was considered significant.

In order to assess the effect of supernatant composition at ratios representative of a blood banking environment, haemolysis values were compared with previously calculated plasma : SAGM ratios observed in Canadian Blood Services WBF and BC processed units (Appendix A)[9]. Briefly, SAGM volumes were calculated for each RCC by measuring mannitol concentration. Two lots of plasma volumes were calculated for each RCC unit; one determined using measured albumin concentrations, a second determined by subtracting the calculated volume of SAGM from the total volume of supernatant.

3.3. Results

3.3.1. Series albumin concentration is proportional with sample plasma content

The albumin concentration of prepared storage samples was measured to demonstrate that the series was generated as desired. Measured albumin concentration was found to increase proportionally with RCC plasma content, with only a slight deviation from the expected albumin values calculated using an average serum albumin concentration from the sample group. Greater deviation in measured albumin concentration was observed at higher plasma volumes (SD <30% plasma: 0.11, SD >30% plasma: 0.49).

3.3.2. RCC supernatant composition effects storage associated haemolysis

Average haemolysis values at each different supernatant composition were compared at the time of sample preparation, and after 21 and 42 days of storage. At the time of sample preparation there was no significant difference in haemolysis across supernatant compositions (Figure 3.2). After 21 days of storage, samples stored in 0% plasma, and samples stored in more than 12.5% plasma exhibited elevated haemolysis compared to the rest of the series (0%: 0.37% ± 0.03%, 2.5-10%: 0.29% ± 0.04%, ≥12.5%: 0.41% ± 0.09%. $p < 0.05$) (Figure 3.2). After 42 days of storage, increasing plasma content is directly correlated with increased haemolysis, with the highest and lowest haemolysis values seen at 0% (0.80% ± 0.20%) and 50% (1.39% ± 0.12%) plasma respectively (Figure 3.2). This trend applies to both WBF and BC processed units and units from both male and female donors.

We compared our observations with previously calculated plasma : SAGM ratios in Canadian Blood Services WBF and BC processed RCCs[10]. There was not a significant difference in haemolysis between the supernatant compositions that most resemble WBF and BC processing conditions at any testing point throughout storage ($p > 0.05$) (Figure 3.2). Observed day 42 haemolysis values were increased compared to Canadian Blood Services quality control values[8]; however this was to be expected due to the increased degree of manual handling of the RBCs during sample preparation.

3.3.3. RCC supernatant composition does not influence RBC volume or haemoglobin content

The ratio of plasma : SAGM in each storage sample did not influence the haemolysis levels significantly at any point during storage, in each of the four study groups. It is worth noting, however, that a significant difference in MCV and MCH was observed between samples in different study groups.

3.3.4. Effect of RCC supernatant composition is not mitigated by RCC processing conditions

Day 42 haemolysis values across all supernatant compositions were elevated in WBF RCCs compared to BC RCCs (WBF: 1.18% ±0.17%, BC: 0.93% ± 0.19%, $p<0.05$) (Figure 3.3). The effect of plasma : SAGM ratio on storage haemolysis is similar in WBF and BC components (WBF R^2: 0.90 BC R^2: 0.94, $p>0.05$).

3.4. Discussion

In this study, our aim was to determine if the volume of residual plasma and additive solution contained in stored RCCs, or more specifically the ratio of these two RCC components, had an influence on the *in vitro* quality and storage efficiency, represented by the rate of storage haemolysis. After 21 days of storage, roughly equivalent to the mean duration of RCC storage in prior to transfusion[11], we observed elevated haemolysis values in RBCs stored in 100% additive solution, and in RCCs containing larger volume of plasma (> 12.5%), when compared to other RCCs. Conversely, after 42 days of storage the level of haemolysis appears directly proportional to increasing plasma content. Drawing on our current understanding of RBC biology we may speculate as to what might be driving this trend.

The effects of residual plasma volume are not well understood; the majority of research in this area has focused on the influence of plasma on transfusion outcomes, as opposed to product quality. Increased volume of plasma has been associated with increased risk of TRALI through a number of mechanisms. Firstly, the increased dose of HLAs (Class I and II) for a patient receiving a large volume of residual plasma[1, 2]. Secondly, storage of RCC's in the presence of autologous plasma has been shown to promote the accumulation of bioactive lysoPCs[3]. Recently, plasma

components have been shown to induce haemolysis under storage conditions. RBCs stored in the presence of lipaemic plasma exhibit a greater level of haemolysis than RBCs stored in non-lipaemic plasma[6, 7]. The observed increased haemolysis at day 21 associated with storage in 100% AS may suggest that in short term storage, at low levels, certain components of residual plasma have a protective effect on the RBC's. One study demonstrated the protective effect of plasma on RBCs *in vitro*; survival factors in plasma were found to inhibit programmed cell death induced by the Bak/Bcl-X_L pathway[12]. The detrimental effect of increasing plasma content observed in all groups following 21 days of storage may suggest that the source of injury is something that accumulates during storage, and only reaches harmful levels in the later weeks of a 42-day storage protocol. Storage accumulated sources of injury influenced by the presence of residual plasma may be derived from the plasma itself, such as the previously mentioned lysoPCs, or could possibly be derived from cellular components.

The volume of additive solution used in RBC storage is known to influence *in vitro* RCC characteristics; indeed, the resuspension of RBCs to account for the volume lost is a primary function of additive solutions [13]. Increased additive solution volumes have been reported to decrease the rate of storage haemolysis[14]. One of the functions of a storage additive solution is to act as a dilution buffer; by-products of RBC metabolism released into the extracellular medium will accumulate over time, affecting intercellular concentration gradients and pH balance. It stands to reason that a larger volume of additive solution would provide an increased buffering capacity[14, 15]. Another key function of storage additive solutions is to provide metabolic fuel for the RBCs during storage; glucose and adenine are both present in SAGM[16]. One could speculate that an increased volume of additive solution used to store the same volume of RBCs will reduce storage haemolysis, due to the increased availability of fuel for the RBCs to metabolise.

The haemolysis values observed in this storage study were compared to manufacturing conditions at Canadian Blood Services by measuring supernatant composition in Canadian Blood Services components (Chapter 2 & Appendix A). No significant difference in haemolysis observed between WBF and BC supernatant compositions. This suggests that the increased haemolysis associated with WBF RCCs compared to BC RCCs is not dependent on component separation efficiency and SAGM delivery, but rather some other factor of the processing methods. Despite the observed influences on product quality and transfusion outcome, neither additive solution or residual plasma volume are monitored by blood services. The inconsistent residual plasma data we gathered (Appendix A) is evidence of the need for a standardised method of measurement; if we are to understand the nuances of the hypothermic storage lesion, we must first be confident we know what ends up in the bags.

3.5. References

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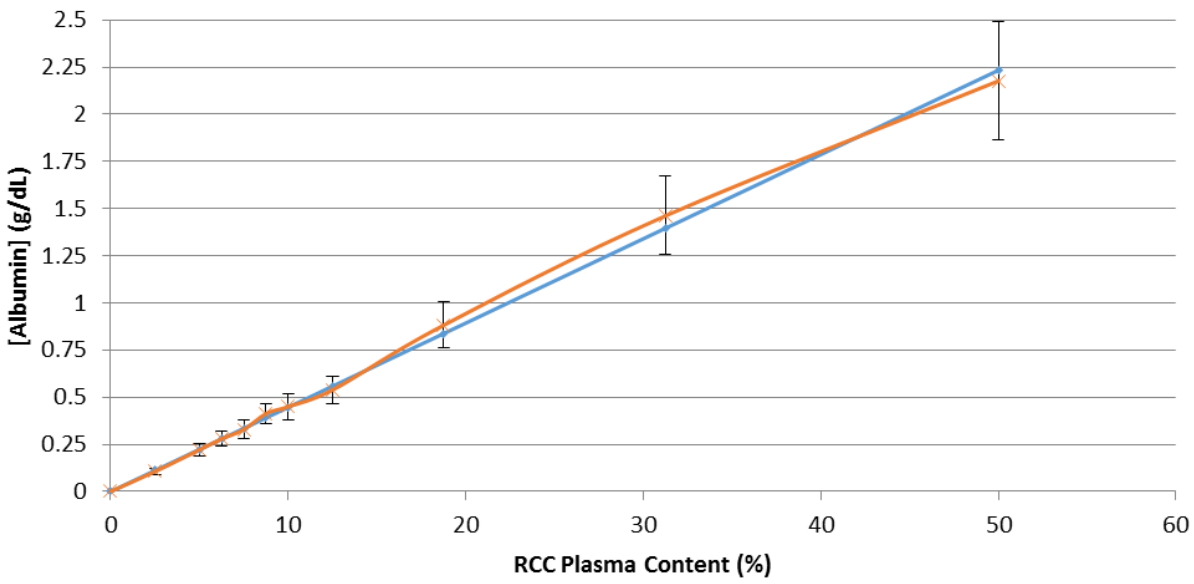
3.6. Figures

Table 3.1 – Plasma & SAGM composition of storage series

Sample	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Plasma (mL)	0.0	0.2	0.4	0.5	0.6	0.7	0.8	1.0	1.5	2.5	4.0
SAGM (mL)	8.0	7.8	7.6	7.5	7.4	7.3	7.2	7.0	6.5	5.5	4.0
% Plasma	0	2.5	5	6.25	7.5	8.75	10	12.5	18.75	31.25	50
Plasma : SAGM	0	0.025	0.05	0.063	0.075	0.088	0.1	0.125	0.188	0.313	0.5

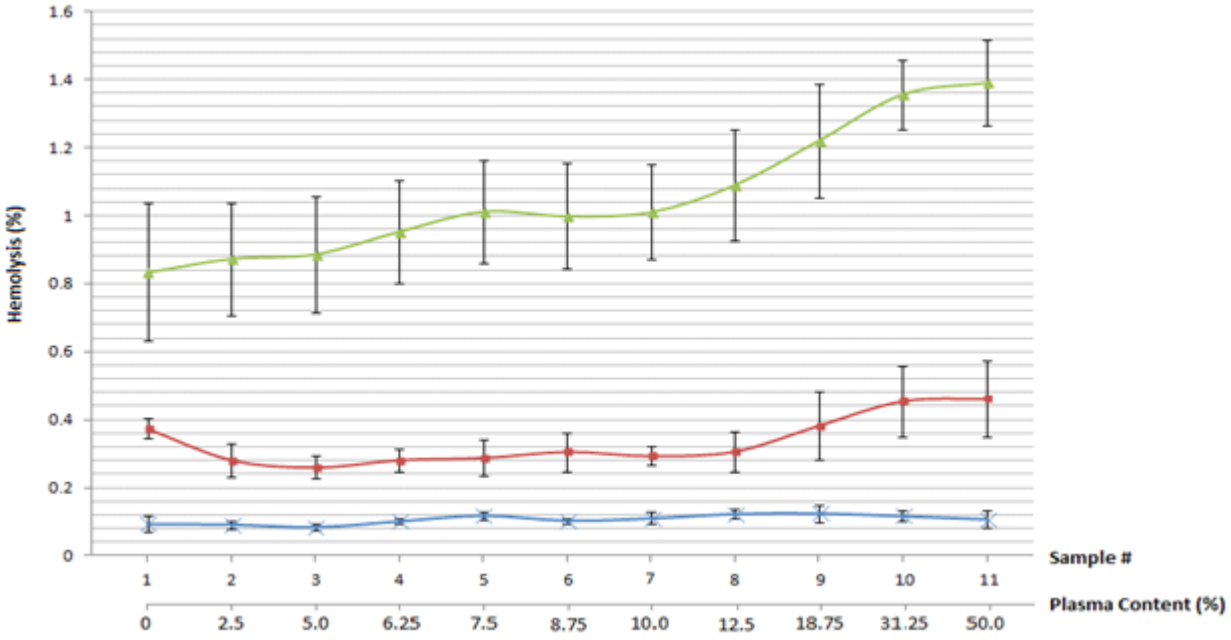
Plasma and SAGM composition of storage samples in each series. Previously observed plasma : SAGM ratios ranged between 0.06-0.14 for WBF processed units, and between 0.11-0.12 for BC processed units, calculated using residual plasma volumes measured using two different techniques.

Figure 3.1 – Validating plasma content of samples using albumin concentration



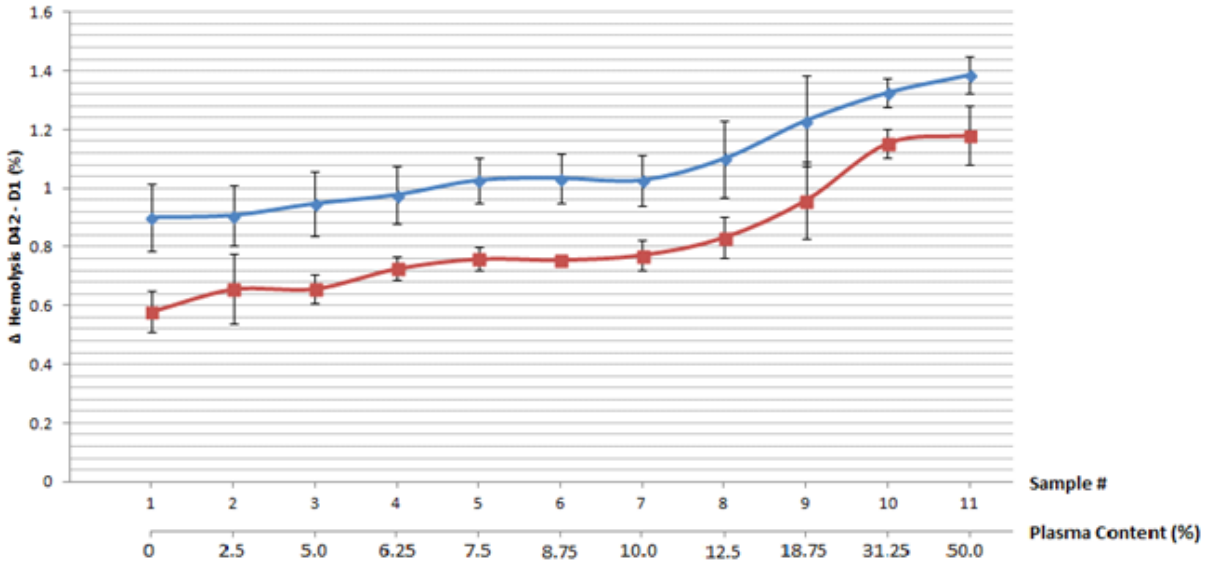
The average measured albumin concentration at each intended plasma level (orange line) was compared to the expected albumin concentration (blue line), calculated using an average serum albumin concentration from the sample group. RCC plasma content is displayed on a scaled axis from 0-50%.

Figure 3.2 – RCC supernatant composition influences storage haemolysis



The average haemolysis across all storage series is shown at the time of sample preparation (D1, blue line), and after 21 (D21, red line) and 42 (D42, green line) days of storage. Plasma content is displayed on a fixed axis along with the corresponding sample number. Previously observed plasma : SAGM ratios ranged between 0.06-0.14 for WBF processed units, and between 0.11-0.12 for BC processed units, calculated using residual plasma volumes measured using two different techniques (appendix A).

Figure 3.3 – RCC processing technique and supernatant composition influence storage haemolysis



The overall storage associated haemolysis for each sample was calculated by subtracting day 1 haemolysis values from day 42 haemolysis values. Results separated based on component processing method; WBF (blue line) or BC (red line).

Chapter 4

Donor Sex and Age Influence Pre-storage and Post-storage RBC Characteristics

4.1. Introduction

The screening and selection of donors is an integral part of the modern blood banking system; essential for controlling transmissible diseases and managing the supply of different blood groups. However, even among donors who meet the selection criteria, certain donor characteristics have been found to influence transfusion outcomes[1,2]. A key example of this is the increased risk of transfusion related acute lung injury (TRALI) associated with the transfusion of components from female donors, specifically female plasma products [3]. This has resulted in plasma products from female donors being discarded by some national blood banking agencies. Recently, donor characteristics have been shown to have an influence on the *in vitro* quality of stored red cell concentrates (RCC), as well as the proposed clinical implications[4-6].

Analysis of quality control (QC) data from national blood banking agencies suggests that storage associated haemolysis in hypothermically stored RCCs is partially dependent on the donor characteristics; sex and age[4-6]. In chapter 2 I presented data supporting this hypothesis, generated from Canadian Blood Services QC data. RCC's from female donors exhibited less

haemolysis after 42 days of storage than components from male donors, and RCC storage haemolysis increased with the donors age at the time of donation. In this respect, young female donors represent the donor group with the highest quality RBC's following 42 days of storage. This trend has been observed by several groups[4-6], however a mechanistic basis for this phenomenon has not been reported.

The aim of this study was to investigate the influence of donor characteristics on a range of *in vitro* RCC parameters using Canadian Blood Services QC data and broad selection of donor information. Effectively expanding on the initial observations made regarding donor influence in chapter 2, and providing a more in depth view of our different "types" or donor.

4.2. Materials and Methods

Between June 2011 and October 2014, a total of 28,266 RCC units were selected for expiry QC testing. Using unique donation ID's, we retrieved manufacturing information (collection date, site and RCC processing method) and non-identifiable donor information (sex, age at time of donation and pre-donation donor haemoglobin) for the units contained within the QC database. In addition to the merged QC database, a separate database was compiled using donor information (sex, age at time of donation, pre-donation donor haemoglobin, collection date and site) collected from 823,675 donations from donors aged 17-70, between October 1st 2013 and October 1st 2014. The data was compiled using Excel spreadsheet software (Microsoft, Redmond, WA).[i]

[i] QC testing, pre-donation donor haemoglobin testing and data recording all performed by Canadian Blood Services staff according to standard operating procedures. Construction of both merged databases performed by myself.

4.2.1. Measuring Donor Haemoglobin

Prior to WB collection donor haemoglobin concentration is tested by Canadian Blood Services as part of the routing donor screening and selection process. A capillary blood sample is obtained; the donors fingertip is sterilized and then punctured using a sterile lancet, the sample is collected in a cuvette (DiaSpect Hemoglobin Cuvettes, DiaSpect, Sailauf, Germany). Haemoglobin is determined spectrophotometrically using an automated haemoglobin analyser (DiaSpect Hemoglobin T, DiaSpect), which measures absorbance across 450-750 nm and calculates total haemoglobin using these absorbance values. Donors with a blood haemoglobin concentration <125 g/L are deferred from donating at Canadian Blood Services for the safety of the donor.

4.2.2. Whole Blood Collection and Processing

WB was collected into Macopharma WBF collection sets (DQE 7291 LX Leucoflex MTL1 Quadruple Top and Top System, CPD/SAGM 500 mL, MacoPharma, Tourcoing, France), Macopharma BC collection sets (LQT 7291 LX leucoflex LCR-Diamond Quadruple Bottom and Top System, CPD/SAGM 500 mL, MacoPharma), Fenwal WBF collection sets (CGR6494B, Quad OptiPure Rc 9SBT WB 500 mL, Fenwal, Lake Zurich, IL, USA) and Fenwal BC collection sets (CGR8441B, Quad PackPure WB 500 mL, Fenwal), and processed according to Canadian Blood Services standard operating procedures as previously described (Figure 2.1)[7][i].

[i] WB collection and component processing performed by Canadian Blood Services staff according to standard operating procedures.

4.2.3. Quality Control Testing

The Canadian Standards Association stipulates that at least 1% of manufactured product at each site must be selected for QC testing every month; these units are sent to one of two QC testing labs (Calgary, AB. & Toronto, ON). Testing for level of haemolysis, unit haemoglobin concentration, and haematocrit was performed at product expiry (day 43 after processing), and the units were destroyed following testing, as previously described (Chapter 2 – 2.2.2)[7]. Briefly; unit volume was calculated from unit mass and density (1.06 g/mL) before any sampling was performed. Total haemoglobin (Hb) and haematocrit were measured using an Advia 120 Hematology System (Siemens AG, Erlangen, Germany) and tri-level haemoglobin controls (R&D Plasma Hemoglobin Hematology Controls, R&D Systems, Minneapolis, MN). Supernatant Hb was measured photometrically at 570 nm and 880 nm (Plasma/Low Hb Photometer, HemoCue, Ängelholm, Sweden) against tri-level haemoglobin controls (R&D Plasma Hemoglobin Hematology Controls, R&D Systems). Percent haemolysis was then calculated using the formula Hly = [supernatant Hb x (1 – Hct)]/total Hb.

4.2.4. Assessing the influence of donation frequency on RBC characteristics

In order to assess how frequency of donation influences haemoglobin levels in donors, and *in vitro* characteristics in stored RCCs; the frequency of donation was calculated for each Canadian Blood Services donor contained within our donor information database[i]. The mean number of whole blood donations per donor was calculated to be 2.6 ± 1.4 donations/year; donors were therefore categorised as either low intensity donors (≤2 donations/year) or high intensity donors (≥3 donations/year). Pre-donation haemoglobin for each donation in September 2014 was

[i] Donation frequency calculations performed by myself. I would like to acknowledge Dr. Sheila O'Brien for providing data on average donation frequency per donor at Canadian Blood Services.

compared to the number of donations from that same donor within the preceding 12 months. Using unique donation identification numbers; QC parameters (haemolysis, unit haemoglobin, haematocrit) for those donations selected for QC testing in September 2014 were compared based on how frequently each donor had donated in the preceding 12 months.

4.2.5. Statistical analysis

For each database, statistical analysis was completed using statistical analysis software (SAS 9.1, SAS, Cary, NC. and SPSS 21, IBM, Armonk, NY)[i]. Regression analysis was performed on the donor database (n = 823,675) to quantify correlational associations between donor sex and age donor pre-donation haemoglobin concentration, R^2 values were compared and a p-value of less than 0.0001 was considered significant. To assess correlational associations between donor factors (sex, age and pre-donation haemoglobin concentration) and stored RCC characteristics (unit haematocrit, unit haemoglobin, unit haemolysis), R^2 values were calculated using the QC database (n = 28,266) and a p-value of less than 0.0001 was considered significant. The distribution of each parameter was assessed for normality using measures for skewness and kurtosis; mean and SD were compared for normally distributed parameters, whilst median and range were compared as well as mean and SD for non-normally distributed parameters. In order to evaluate the statistical significance of the influence of each donor factor on the measured RBC characteristics; a random effects mixed model analysis was performed on both sets of data, using unique donor ID as the random effects variable to account for the clustering effect of repeat donors. For each outcome variable, a univariate mixed model with a single predictor was first fitted, other variables were then added to the model to assess the adjusted effect of the initial predictor after potential

[i] Statistical analysis was performed by Qi Long Yi and myself. I would like to thank Qi Long Yi for guidance and help choosing and performing the appropriate statistical tests, and help writing the Statistical Analysis section of this chapter.

confounding from the each added variable. A p-value of less than 0.0001 was considered significant.

To assess the impact of donation frequency on donor pre-donation haemoglobin levels; pre-donation haemoglobin means were compared between high and low intensity donors in each donor group using a non-parametric Pearsons' Chi Squared test, a p-value of $p < 0.0001$ was considered significant. Assessing the impact of donation frequency on stored RCC characteristics, a much smaller sample size of QC data was available for donors within the same time frame (n = 304). For each QC output variable, a univariate mixed model was fitted with donation frequency as the predictor, other variables were then added to assess the adjusted effect of donation frequency with potential confounding from other donor factors. A p-value of $p < 0.01$ was considered significant.

4.3. Results

4.3.1. Donor sex and age and influence RBC characteristics in stored RCCs

Unit haemolysis, unit haematocrit and total unit haemoglobin levels were measured in all RCCs selected for QC testing (n = 28,266); donor groups were further stratified by WB processing method (BC/WBF) to account for confounding due to the influence of manufacturing processes on RCC characteristics observed in chapter 2 (Table 2.1, Figure 2.2). Unit haemolysis was found to be lower in RCCs from females compared to males, whilst increasing age was associated with increased unit haemolysis in both male and female RCCs (Δ haemolysis in old vs young females: 0.01%, $p < 0.0001$, Δ haemolysis in old vs young males: 0.02%, $p < 0.0001$). Mean unit haematocrit, and mean total unit haemoglobin were both found to be significantly lower in RCCs from female donors compared to units from male donors (Table 4.1, Figures 4.1 & 4.2, $p < 0.0001$). Increasing donor age did not have a significant influence on unit haematocrit in female donors

age 18-40. However, in female donors age 40 years and over, increasing donor age correlated with increasing unit haematocrit (Figure 4.1, R^2: 0.932, $p < 0.0001$). Donor age did not have a significant influence on haematocrit in units from male donors (Figure 4.1). Unit haemoglobin increased steadily with donor age in female donors and decreased steadily with donor age in male donors (Figure 4.2, male: $R^2 = -0.65$, $p < 0.0001$, female: $R^2 = 0.62$, $p < 0.0001$), with WBF processed RCCs containing more haemoglobin per unit than BC processed RCCs due to larger mean unit volume.

4.3.2. Donor sex and age influence donor haemoglobin levels

Donor haemoglobin is assessed prior to donation to ensure the donor meets the eligible donation requirements. The influence of donor sex and age on donor haemoglobin levels were assessed using pre-donation haemoglobin levels collected from 823,675 potential donors between October 2013 and October 2014. We observed lower average haemoglobin concentrations in female donors compared to male donors across all ages (Table 4.2 & Figure 4.3; 140 ± 10 g/dL vs. 154 ± 13 g/dL respectively, $p < 0.0001$). Between the male and female donor populations, donor age at time of donation had opposing effects on pre-donation haemoglobin values. Young female donors were found to have the lowest mean haemoglobin concentration of the four donor groups, whilst young males exhibited the highest pre-donation haemoglobin values (Table 4.2 & Figure 4.3; 139 ± 10 g/dL vs. 156 ± 13 g/dL respectively, $p < 0.0001$). In the male population, increasing donor age was associated with a steady decline in pre-donation haemoglobin concentration (Figure 4.3, $R^2 = 0.98$, $p < 0.0001$). Donor age in females did not have a significant influence on pre-donation haemoglobin level in donors ages 17-45 ($p > 0.001$). In female donors aged 45 and over, increasing donor age correlated with increasing donor haemoglobin (Figure 4.3, $R^2 = 0.57$, $p < 0.0001$), with the highest rate of increase between 45 and 53 years of age (45 years = 138 ± 7 g/dL, 53 years = 141 ± 10 g/dL, $R^2 = 0.66$, $p < 0.0001$).

4.3.3. Donor pre-donation haemoglobin concentration influences RCC haematocrit and haemoglobin content

Donor haemoglobin levels correlated positively with unit haematocrit and total unit haemoglobin level in both male and female donations (Figures 4.4 & 4.5, male: $R^2 = 0.97$, $p < 0.0001$, female: $R^2 = 0.86$, $p < 0.0001$). RCCs from male donors exhibited greater unit haematocrit and total unit haemoglobin values than female donations even in donors with the same pre-donation haemoglobin levels (Figures 4.4 & 4.5; Male donor @ 150 g/L pre-donation Hb: 64 g/unit ± 3.2 g/unit, 0.67 L/L ± 0.03 L/L. Female donor @ 150 g/L pre-donation Hb: 58 g/unit ± 3.8 g/unit, 0.66 L/L ± 0.03 L/L).

4.3.4. Donor pre-donation haemoglobin concentration influences haemolysis in stored RCCs

The association between pre-donation haemoglobin levels and RCC storage haemolysis was found to be dependent on RCC processing method. In WBF processed RCCs, increasing pre-donation haemoglobin was associated with decreased storage haemolysis in both male and female donors (Figure 4.6, male: $R^2 = 0.40$, $p < 0.0001$, female: $R^2 = 0.22$, $p < 0.0001$). Pre-donation haemoglobin levels were not found to be associated with storage haemolysis in BC processed RCCs. We observed the same trend in haemolysis associated with donor sex and age as described previously (Chapter 2, Figures 2.2 & 2.3); units from female donors exhibit less haemolysis at the end of storage than those from male donors, whilst increasing donor age correlated with increasing levels of haemolysis in both sexes.

4.3.5. Frequency of donation influences pre-donation haemoglobin values

Frequency of donation was found to have a significant influence on pre-donation donor haemoglobin levels, with more frequent donations associated with lower pre-donation donor haemoglobin (Figure 4.7, $p < 0.0001$ between high and low intensity donors from each donor group). The difference in pre-donation haemoglobin was greater between low intensity and high intensity male donors than between low and high intensity female donor. The largest difference in pre-donation haemoglobin was observed between low intensity and high intensity young male donors.

4.3.6. The influence of donation frequency on the characteristics of stored RCCs is dependent on donor factors and WB processing methodology

In vitro RCC characteristics were compared in donations from high intensity donors (3 or more donations in the preceding 12 months) and low intensity donors (2 or fewer donations). Unit haematocrit was found to be significantly lower in high intensity donors compared to low intensity donors (Table 4.3, $p < 0.05$); this pattern was observed in all donor groups and processing methods. The influence of donation frequency on haemolysis values in stored RCC was shown to be dependent on both donor factors (age and sex) and component processing method (Table 4.3). In the female donor population, high intensity donors exhibited increased post-storage RCC haemolysis than their low intensity counterparts. Within the male donor population, we observed the opposite relationship; high intensity male donors exhibited decreased post-storage RCC haemolysis than low intensity male donors. Donation frequency was not found to have a significant influence on RCC unit haematocrit regardless of donor factors or processing method.

4.4. Discussion

Previously we discussed the influence of donor sex and age on RCC haemolysis, reporting lower storage haemolysis levels in RCCs from female donors compared to male donors, and a correlation between increasing donor age and greater RCC haemolysis (Chapter 2, Figures 2.2 & 2.3)[4]. In this study we take a closer look at the *in vitro* characteristics of stored RCCs, using large scale QC analysis to investigate unit haematocrit and total unit haemoglobin as well as unit haemolysis. Our aim was to determine how these characteristics were influenced by donor sex and age, whilst introducing two more donor factors; donor pre-donation haemoglobin level, and frequency of donation. We observed lower pre-donation donor haemoglobin levels, unit haemolysis, unit haematocrit and total unit haemoglobin in female donors compared to male donors. The effect of donor age on pre-donation haemoglobin level, unit haematocrit and total unit haemoglobin was dependent on donor sex. We observed a positive correlation between pre-donation haemoglobin levels and both unit haematocrit and total unit haemoglobin, however the relationship between pre-donation haemoglobin levels and unit haemolysis was found to be dependent on component processing method. Frequency of donation was shown to have a sex dependent effect on donor pre-donation haemoglobin levels, with frequency of donation having the most significant effect on the pre-donation haemoglobin levels of young male donors (18-40 years). Frequency of donation was not found to have a statistically significant influence on unit haematocrit or unit haemolysis.

Donor factors have long been associated with clinical outcomes in blood transfusions; ranging from fundamental transfusion practices such as ABO/Rh blood group matching and pathogen screening, to more current concerns such as the increased risk of TRALI associated with female plasma products[3]. Recently it has come to light that donor factors might also influence the *in vitro* quality of stored blood products[4-6]. Donor sex, age and ethnicity have been reported to

influence the degree of RBC haemolysis in stored RCCs; units from female donors exhibit less storage haemolysis than those from male donors, whilst increasing donor age is associated with increased haemolysis. The data we report here and in chapter 2 follows these trends. The mechanisms behind these observations have not been reported, however, a number of hypotheses have been suggested.

Increasing osmotic fragility in RBCs as a function of human aging was first reported by Detraglia *et al*[8], whilst osmotic fragility as a function of sex was first reported in chickens in 1966[9], then later in humans[10]. It was suggested that the increased osmotic fragility is due to a decrease in the surface area to cell volume ratio of circulating RBCs. In turn, this loss of shape was suggested to be due to RBC senescence[11, 12]. Here we present data showing how donor sex and age influence donor haemoglobin levels (Table 4.2, Figure 4.3), RCC haematocrit (Figure 4.1) and RCC haemoglobin content (Figure 4.2). We observed that the most significant change in donor pre-donation haemoglobin and unit haematocrit in female donors occurs around 50 years of age. Several groups have reported increased mechanical fragility in both fresh [13] and stored[10] RBCs from pre-menopausal women. We previously reported increased storage haemolysis in post-menopausal women compared to pre-menopausal women[Chapter 2; Figure 2.3, 3]. It is possible that the increase in pre-donation donor haemoglobin and haematocrit during this age range also corresponds to the cessation of regular blood loss due to menopause. It has been suggested that menstrual blood loss in pre-menopausal females results in a younger population of circulating RBCs, with decreased osmotic fragility compared to older RBCs, accounting for the reduced risk of cardiovascular disease observed in pre-menopausal women[13]. One could speculate that this younger population of RBCs could also contribute to the low storage haemolysis associated with young female donors.

Other proposed mechanisms behind the influence of donor characteristics on RBC morphology and haemolysis attribute this phenomenon to the effects of sex hormones, namely progesterone and testosterone. One hypothesis suggests that the protective effect of progesterone contributes to the decreased RBC fragility associated with pre-menopausal females[14]. The decrease in women's progesterone levels associated with menopause coincides with an increase in RBC mechanical fragility and haemolytic potential. RBCs are thought to be an important transporter of progesterone, which has been reported binding to the RBC membrane[15]. Progesterone has also been reported to have a protective effect on stored RBCs during storage[15, 16], on the contrary, ovariectomy in female mice has been shown to have no influence on RBCs fragility[17]. Another hypothesis holds that the detrimental effects of testosterone on RBCs is a key factor in the increased storage haemolysis associated with male donors compared to female donors[17]. Testosterone has been shown to promote erythropoiesis[18], which could explain the increased haematocrit and haemoglobin levels we observed in male donors compared to female donors. Bachman *et al* reported increased haematocrit and patient haemoglobin levels in older men (≥65 years) receiving testosterone administration compared to a control group. They observed increased erythropoietin levels and decreased ferritin and hepcidin levels in patients receiving testosterone, leading them to suggest that testosterone stimulates erythropoiesis through erythropoietin regulation[19]. As testosterone levels decrease with age in males over 30[20], this theory may explain the decrease in pre-donation haemoglobin levels with age in males over 30. However, this does not account for the correlation between age and pre-donation haemoglobin we observed in male donors under the age of 30, when testosterone levels are steady. Kanias *et al* proposed that the action of testosterone during erythropoiesis may also influence RBC rheology, leading to increased susceptibility to stress damage during storage[17]. They reported a reduced haemolytic response to osmotic and oxidative stress in RBCs from orchiectomised mice compared to control males; observing a stress response similar to the RBCs of female mice, and reported increased haemolysis in orchiectomised mice receiving testosterone repletion for 32 prior to

sampling. Interestingly, they did not observe an increased haemolytic response in RBCs stored in the presence of testosterone compared to a control group. If the action of testosterone during erythropoiesis results in a male RBC with a predisposition for haemolysis, this could account for the increased haemolysis in RCCs from male donors compared to female donors. However, this hypothesis does not explain the observed effect of donor age on RBC storage haemolysis in male donors. As testosterone levels decrease with age in males over 30, one would expect to see a resulting decrease in RBC storage haemolysis. As this is not the case based on current observations, this suggests that there are other factors involved in the age response in male donors.

As evidenced by the contrasting observations and ideas; we are far from reaching a consensus on the mechanisms behind the influence of donor factors on RBC storage. The observations we report here demonstrate a complex relationship between donor factors and RCC characteristics, specifically unit haematocrit and total haemoglobin content. How the observed physiological differences relate to the haemolytic propensity of stored cells from different donor groups is not clear, and warrants further research. More data on the biochemical and morphological differences between fresh and stored RBCs from different donor groups is required if we are to accurately describe the mechanisms underlying the influence of donor age and sex on RBC storage injury.

4.5. References

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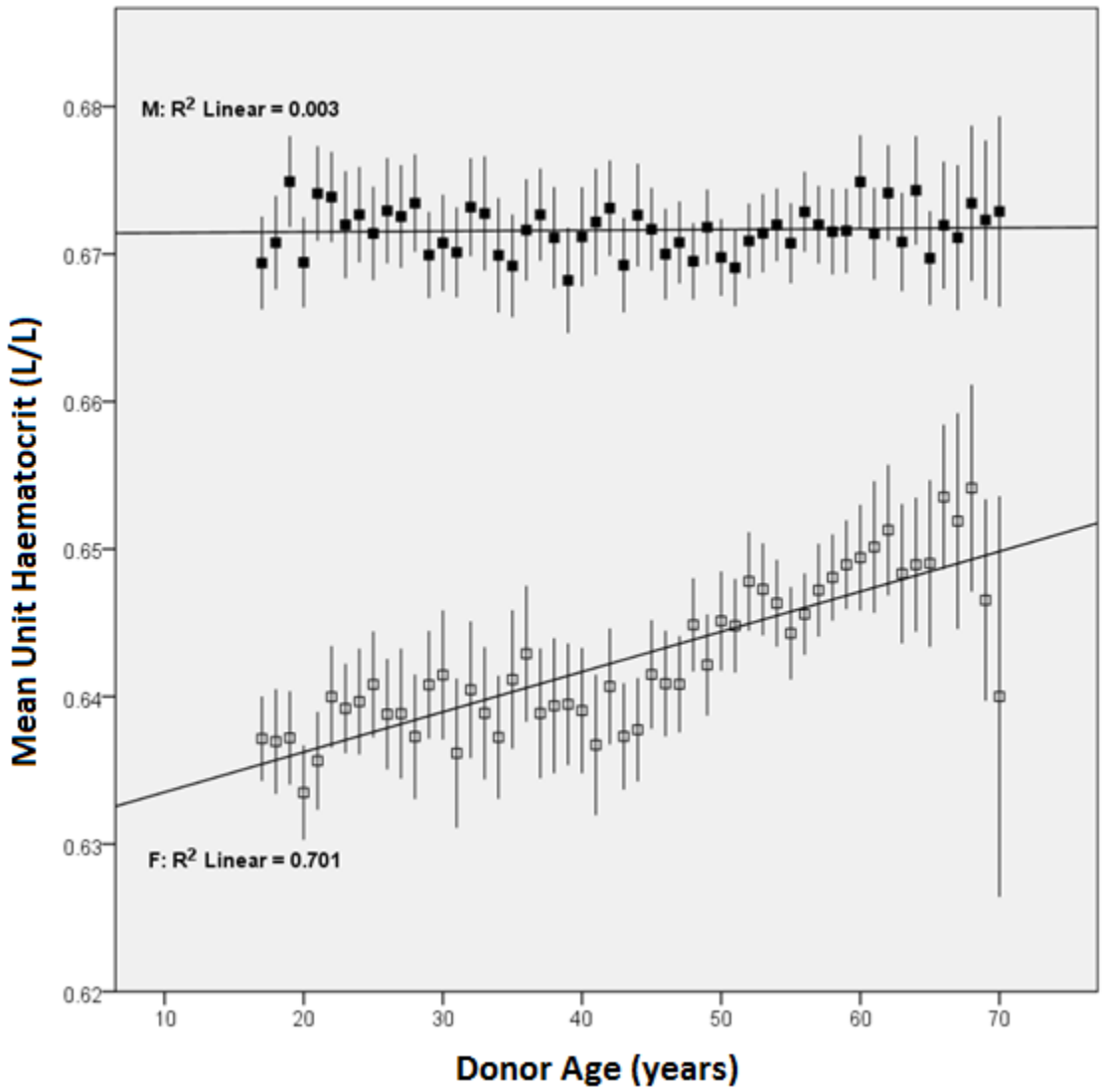
4.6. Tables & Figures

Table 4.1: Summary of post-storage RCC characteristics for donor groups

Donor Age Groups	**Unit Haemolysis** (%)	**Unit Haematocrit** (L/L)	**Unit Haemoglobin** (g/unit)
Females 18-40 (n = 7366)	0.20 (0.05 – 3.11)*	0.64 (± 0.03)*	53.1 (± 6.6)*
Females ≥ 60 (n = 4373)	0.21 (0.05 – 2.86)*	0.65 (± 0.03)*	54.6 (± 6.6)*
Males 18-40 (n = 9604)	0.24 (0.04 – 5.53)*	0.67 (± 0.03)*	62.1 (± 7.0)*
Males ≥ 60 (n = 6884)	0.26 (0.04 – 7.59)*	0.67 (± 0.03)*	60.8 (± 7.3)*

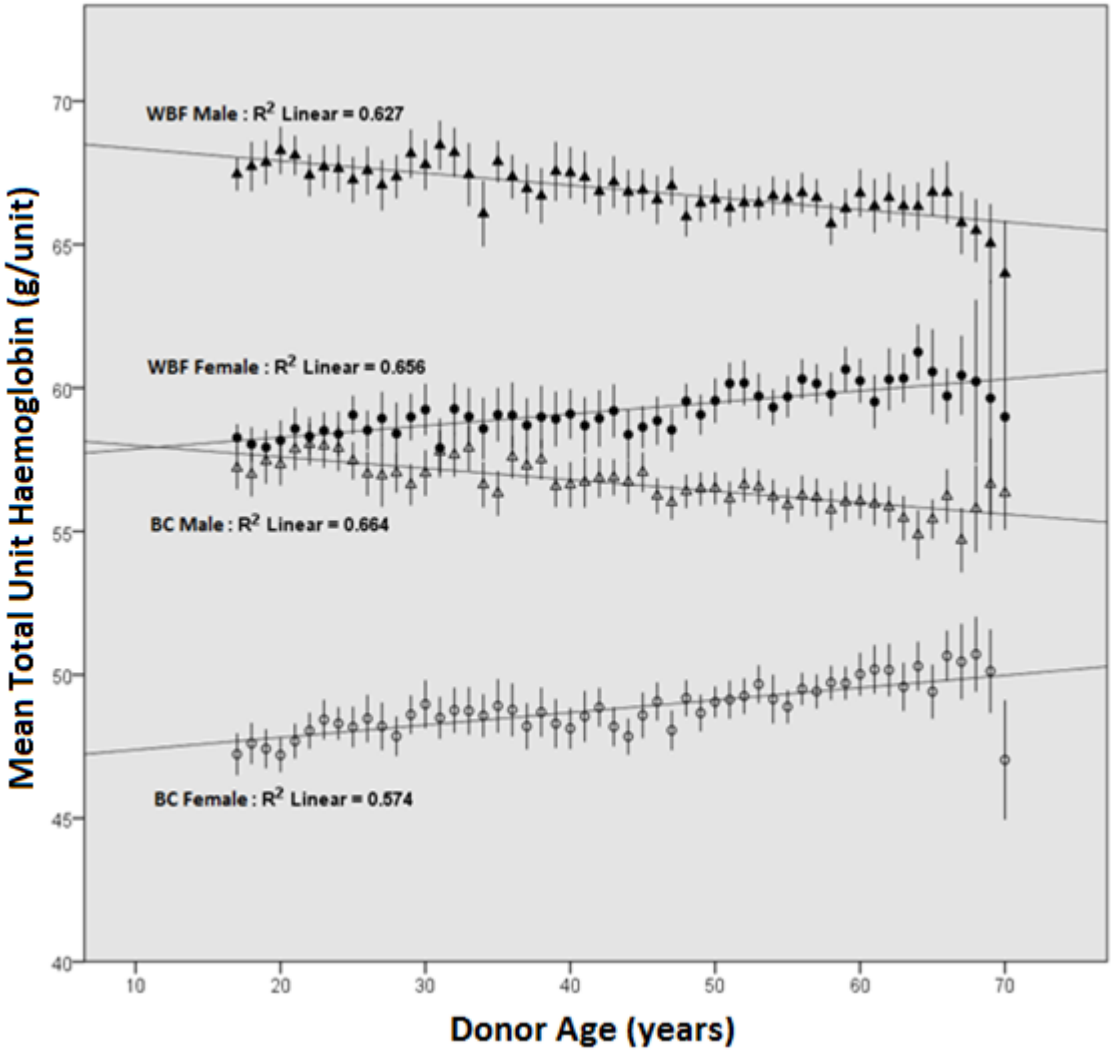
Data collected within 24 hours of product expiry (following 42 days of storage at 4±2°C). Mean (± 1SD) reported for unit haemoglobin, and median (range) reported for unit haematocrit and haemolysis. * Significant differences in mean haemolysis, haematocrit and unit haemoglobin were observed between all groups ($p<0.01$). N = 28,227.

Figure 4.1: Donor sex and age at time of donation influence RCC haematocrit



Mean unit haematocrit plotted against donor age, categorised based on donor sex; male (■) and female (□). RCC haematocrit values measured within 24 hours of product expiry (following 42 days of storage at 4±2°C). Error bars represent 95% confidence interval. N = 28,227.

Figure 4.2: Donor sex and age and RCC processing methodology influence RCC haemoglobin content



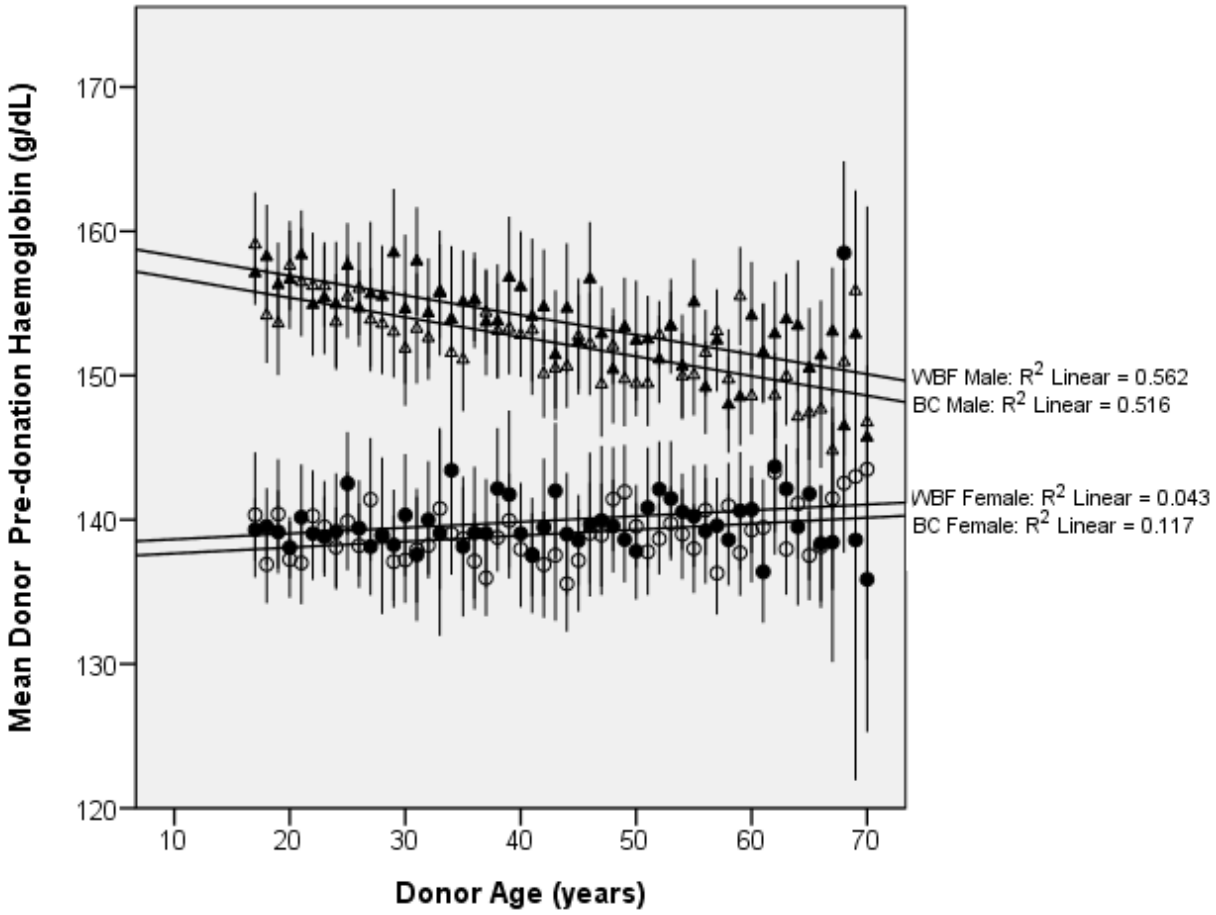
Mean unit haemoglobin plotted against donor age, categorised into donor/manufacturing groups; WBF male (▲), BC male (Δ), WBF female (●), BC female (○). RCC haemoglobin values measured within 24 hours of product expiry (following 42 days of storage at 4±2°C). Error bars represent 95% confidence interval. N = 28,227.

Table 4.2: Pre-donation haemoglobin levels in donor groups

Donor Group	**Pre-Donation Haemoglobin** (g/dL)
Females 18-40 (n = 159,473)	139 (± 10)*
Females ≥60 (n = 50,811)	140 (± 10)*
Males 18-40 (n = 168,026)	156 (± 13)*
Males ≥60 (n = 81,468)	151 (± 13)*

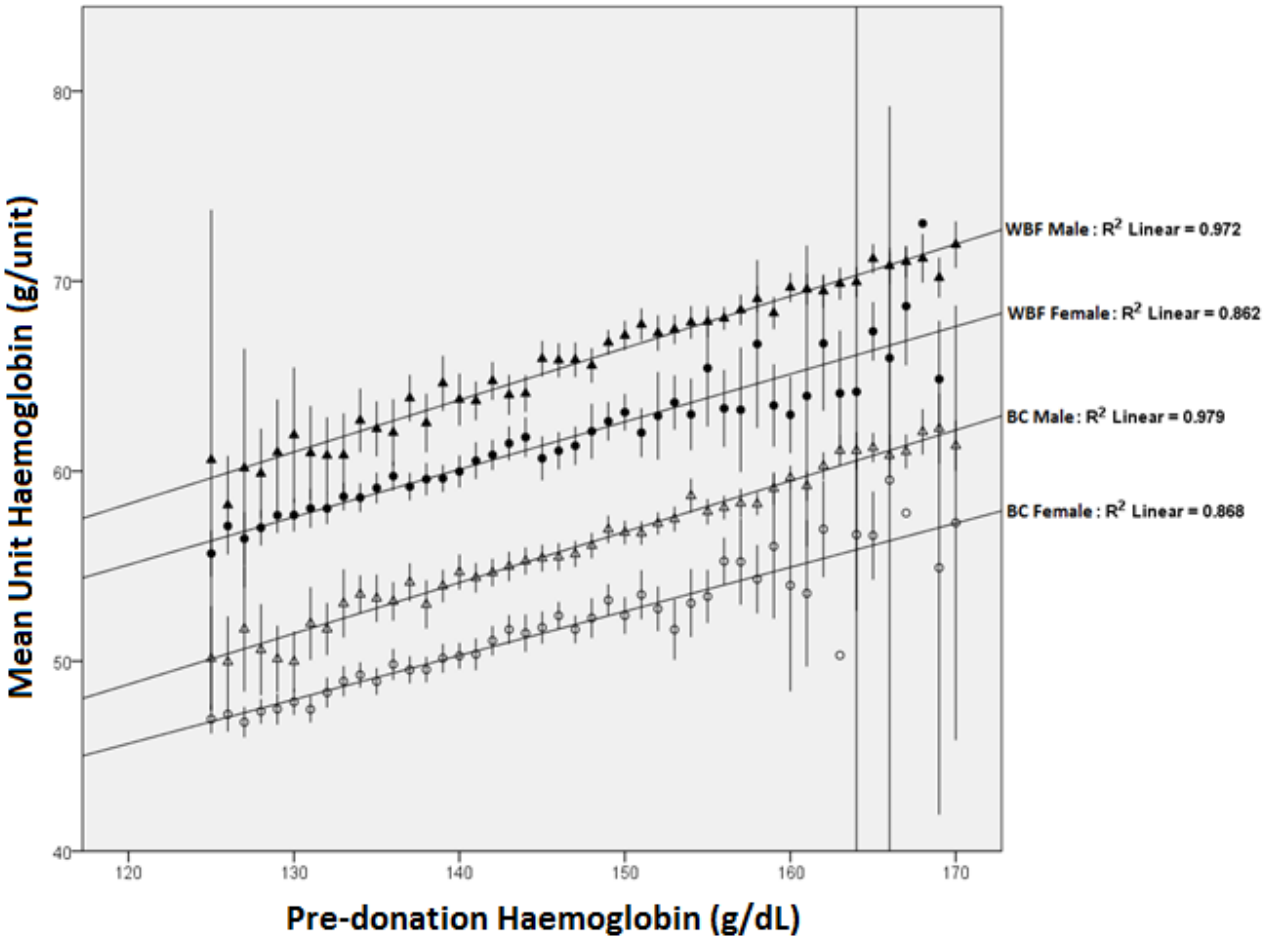
Mean (± 1 SD) pre-donation haemoglobin values for each donor group. Total sample size = 459,778. Data collected from 2013.11 to 2014.10. * Significant difference in mean pre-donation haemoglobin was observed between all groups ($p<0.001$).

Figure 4.3: Donor sex and age influence pre-donation haemoglobin



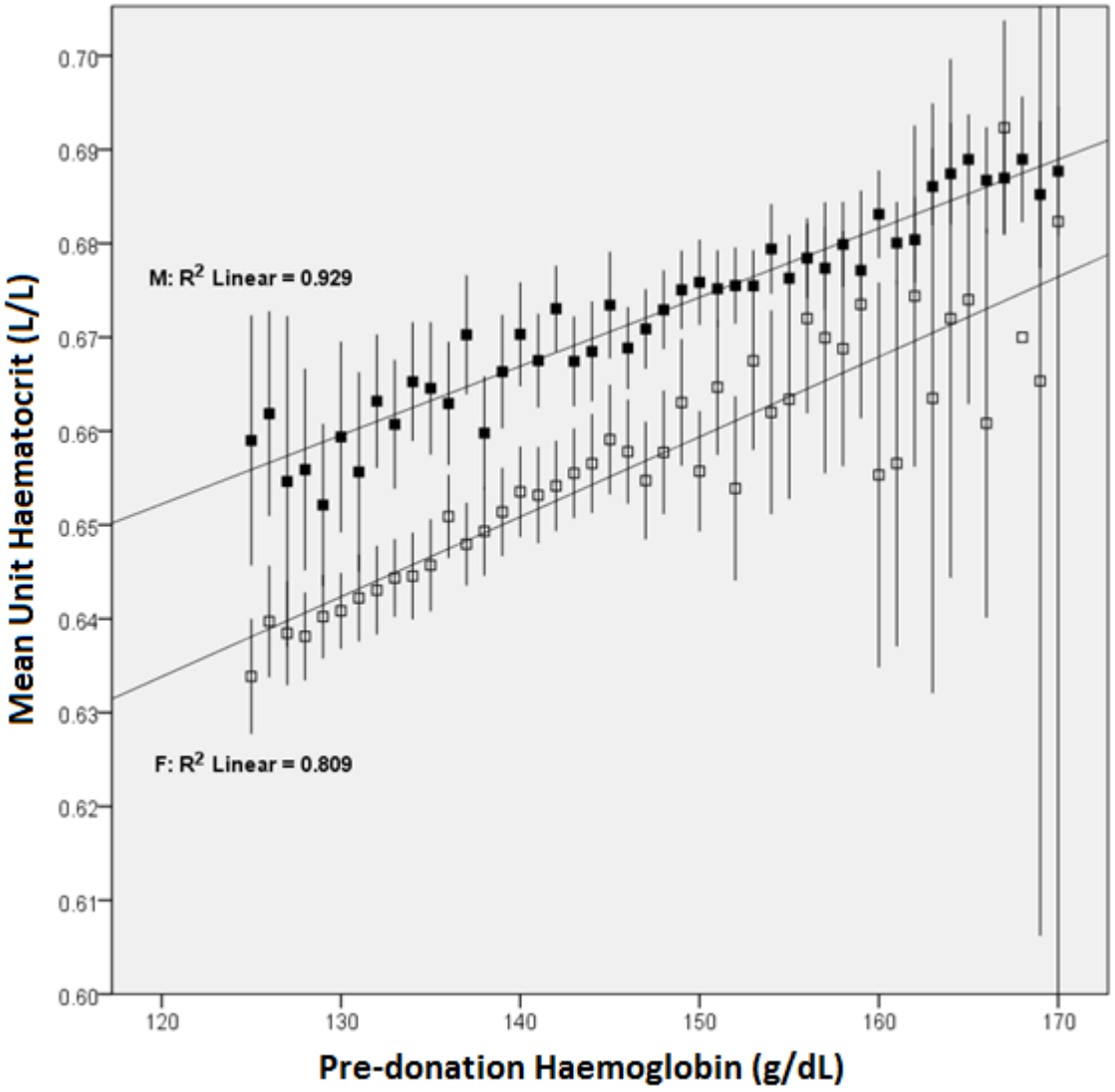
Pre-donation haemoglobin values collected from 459,778 donors between October 2013 and October 2014. Mean pre-donation haemoglobin plotted against donor age, and categorised based on donor sex.

Figure 4.4: Donor haemoglobin levels influence RCC haemoglobin content



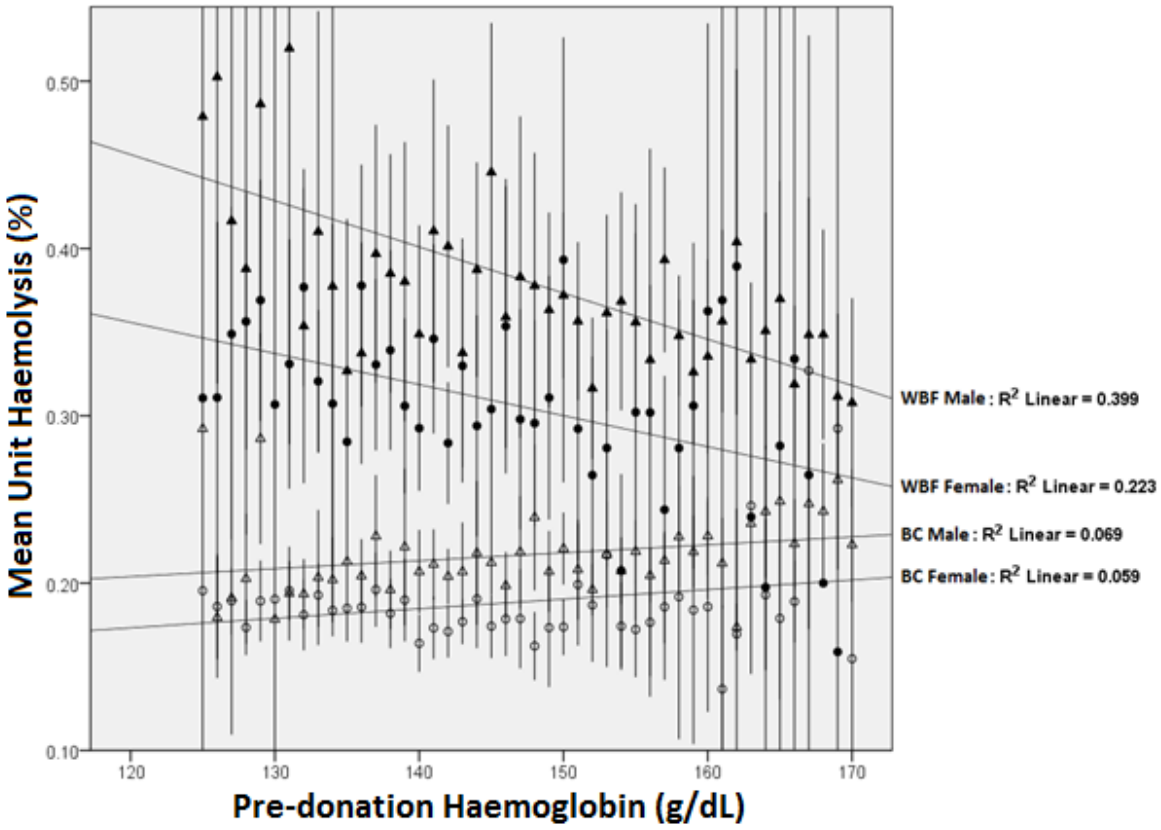
Mean unit haemoglobin plotted against donor pre-donation haemoglobin values, categorised into donor/manufacturing groups; WBF male (▲), BC male (Δ), WBF female (●), BC female (○). RCC haemoglobin values measured within 24 hours of product expiry (following 42 days of storage at 4±2°C). Error bars represent 95% confidence interval. N = 7,162.

Figure 4.5: Donor haemoglobin levels influence RCC haematocrit



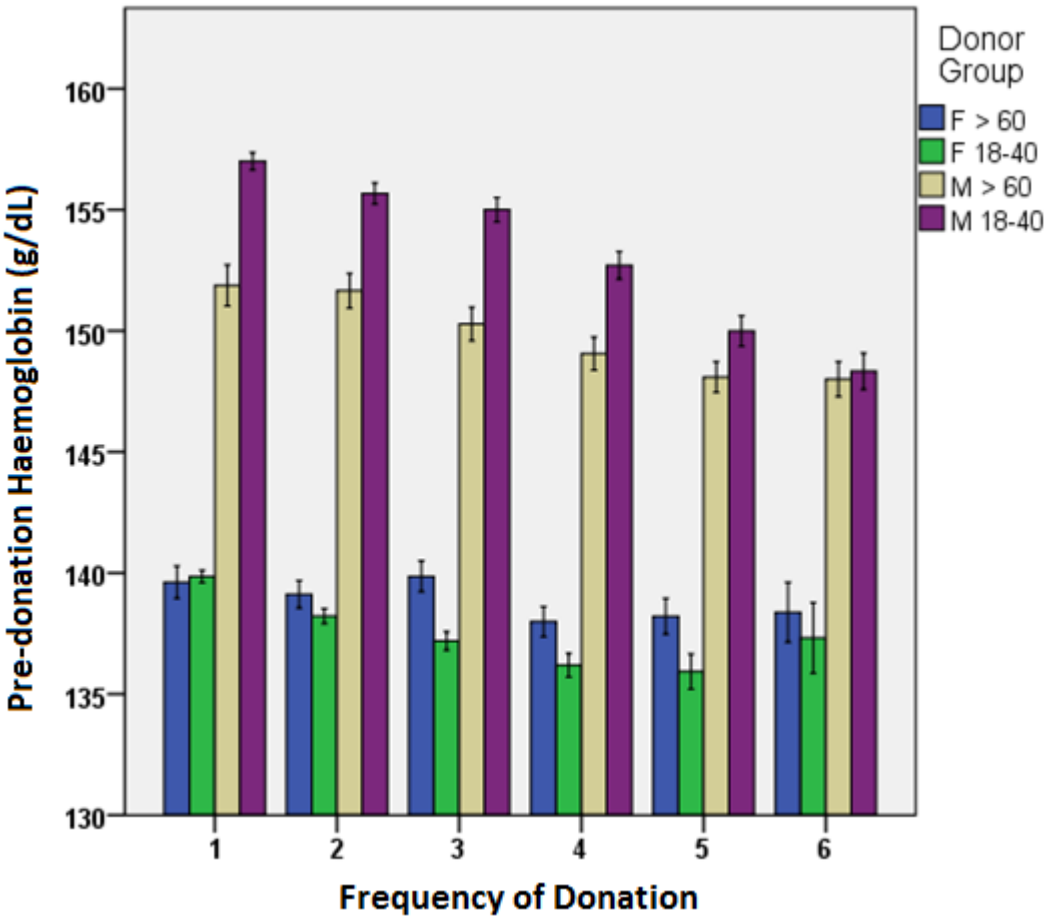
Mean unit haematocrit plotted against donor pre-donation haemoglobin values, categorised based on donor sex; male (■) and female (□). RCC haematocrit values measured within 24 hours of product expiry (following 42 days of storage at 4±2°C). Error bars represent 95% confidence interval. N = 7,162.

Figure 4.6: The influence of pre-donation haemoglobin on post-storage haemolysis in RCCs is dependent on RCC processing method



Mean unit haemolysis plotted against donor pre-donation haemoglobin values, categorised into donor/manufacturing groups; WBF male (▲), BC male (Δ), WBF female (●), BC female (○). RCC haemolysis values measured within 24 hours of product expiry (following 42 days of storage at 4±2°C). Error bars represent 95% confidence interval. N = 7,162.

Figure 4.7: Frequency of donation influences pre-donation haemoglobin levels



Mean pre-donation haemoglobin levels for Canadian Blood Services donors aged 18-40 and >60 in September 2014 (n = 36,105). Plotted as a function of donation frequency for each donor during the period between September 2013 and September 2014, inclusive. Error bars represent 95% confidence interval.

Table 4.3: The influence of donation frequency on stored RCC characteristics is dependent on donor factors and component processing conditions

	Low Intensity Donors			High Intensity Donors		
	Haemolysis (%)	**Unit Haemoglobin** (g/unit)	**Unit Haematocrit** (L/L)	**Haemolysis** (%)	**Unit Haemoglobin** (g/unit)	**Unit Haematocrit** (L/L)
F 18-40	0.23 ± 0.17	53.0 ± 6.1	0.66 ± 0.02	0.25 ± 0.13	50.9 ± 5.6	0.65 ± 0.02
BC (n = 20)	0.17 ± 0.06	49.8 ± 3.0	0.65 ± 0.02	0.22 ± 0.10	49.1 ± 4.5	0.65 ± 0.02
WBF (n = 29)	0.37 ± 0.25	60.5 ± 4.6	0.66 ± 0.02	0.34 ± 0.22	57.8 ± 2.5	0.66 ± 0.02
F ≥ 60	0.24 ± 0.11	56.4 ± 3.4	0.66 ± 0.01	0.30 ± 0.05	54.4 ± 3.9	0.66 ± 0.02
BC (n = 18)	0.21 ± 0.09	54.9 ± 5.2	0.65 ± 0.01	0.22 ± 0.04	52.9 ± 5.1	0.66 ± 0.02
WBF (n = 11)	0.26 ± 0.10	62.7 ± 1.6	0.68 ± 0.01	0.31 ± 0.05	57.6 ± 2.8	0.66 ± 0.02
M 18-40	0.27 ± 0.18	62.8 ± 4.0	0.69 ± 0.02	0.25 ± 0.13	61.1 ± 3.7	0.69 ± 0.01
BC (n = 101)	0.26 ± 0.16	59.1 ± 5.0	0.69 ± 0.02	0.18 ± 0.06	56.9 ± 3.6	0.69 ± 0.01
WBF (n = 59)	0.29 ± 0.20	69.1 ± 3.1	0.69 ± 0.02	0.33 ± 0.13	68.1 ± 3.8	0.69 ± 0.02
M ≥ 60	0.28 ± 0.11	61.59 ± 3.9	0.70 ± 0.02	0.25 ± 0.11	59.5 ± 4.8	0.69 ± 0.02
BC (n = 44)	0.24 ± 0.09	57.5 ± 4.8	0.69 ± 0.03	0.21 ± 0.07	55.5 ± 4.6	0.68 ± 0.02
WBF (n = 22)	0.37 ± 0.08	69.9 ± 3.0	0.71 ± 0.01	0.33 ± 0.13	67.8 ± 5.0	0.71 ± 0.01

Average unit haematocrit, haemoglobin and haemolysis values reported for donations in September 2014 from high frequency and low frequency male and female donors. High frequency donors were classified as having donated more than 3 times in the preceding 12 months, and low frequency donors having donated less than 3 times in the same period. Mean (± 1SD) reported for unit haemoglobin, unit haematocrit and haemolysis.

Chapter 5

Donor Sex and Age at Time of Donation Influence *In Vitro* and *In Vivo* RBC Morphology

5.1. Introduction

The influence of donor factors, such as sex and age, on RCC product quality and clinical transfusion outcomes has garnered recent interest within the field of transfusion medicine. Multiple groups have reported significantly decreased haemolysis in units from pre-menopausal female donors when compared to donations from other donor populations[1, 2]. A number of theories have been suggested to explain this trend[3-5], based on known physiological differences between males and females and their circulating RBCs, however no confirmed mechanisms have been reported. Due to the association between the observed differences in storage haemolysis and female menopausal age/status, several hypotheses attribute the phenomenon to the action of sex hormones[3, 4]. Alternatively, another theory suggests that regular blood loss in pre-menopausal female donors results in a younger population of circulating RBCs, which are less susceptible to stress and haemolysis during storage[5].

The average *in vivo* lifespan for RBCs in healthy adults is around 120 days, with an average turnover rate of 200 billion RBCs per day[6]. RBCs in circulation should therefore be considered a fluctuating population of cells of all different ages. The current model of *in vivo* RBC senescence describes a number of morphological and biochemical changes in the cell as it ages, eventually resulting in recognition and clearance by the mononuclear phagocyte system[7]. Cell shrinkage and loss of surface area have been reported in aged erythrocytes, resulting in reduced deformability compared to younger cells[7, 8]. Characterisation of human RBCs based on cell density suggests that increasing cell density may also be a function of cell age[9].

Along with haemolytic propensity, we previously reported a number of co-occurring differences in RCCs from populations of male and female donors of different ages; we observed lower unit haematocrit and total unit haemoglobin values in young female donors compared to the rest of the donor population (Chapter 4). Interestingly, the observed differences in unit haematocrit and total unit haemoglobin were present in donors with the same pre-donation haemoglobin levels. For example; female donors with a pre-donation haemoglobin concentration of 150 g/dL were found to have lower average unit haematocrit and total unit haemoglobin than male donors with the same pre-donation haemoglobin concentration (female = 65 L/L, 52 g/unit, male = 67 L/L, 58 g/unit). One possible explanation for these observations is that there in a difference in RBC volume and density based on donor age and sex, which could in turn be associated with the age of circulating RBCs.

The objective of this study is to determine the effects of donor sex and age on pre-storage and post-storage RBC characteristics: volume and density and haemoglobin content. Our aim is to further define the role of *in vivo* cell senescence on the haemolytic propensity of stored RBCs from different donor populations.

5.2. Materials and Methods

For this investigation, a mixed approach including retrospective data analysis and a prospective RBC storage experiment was used. Analysis of Canadian Blood Services quality management program (QMP) data was performed[i]; comparing the mean RBC volume, RBC haemoglobin content and RBC haemoglobin concentration in RCCs from four different donor populations (females aged 18-40, females aged ≥60, males aged 18-40, males aged ≥60) throughout storage. For the storage study, RCCs categorised using the same four donor groups were assessed throughout a 42-day storage period for RBC density, RBC size and RBC haemoglobin concentration.

5.2.1. Quality Monitoring Program

The Canadian Blood Services QMP assessed RCCs collected and processed according to the current Canadian Blood Services SOPs as previously described (Chapter 2 – 2.2.1, Figure 2.1)[10][ii].

RBC units were shipped to a CBS laboratory in Edmonton, Alberta, Canada, using J-82 shipping boxes containing ice packs and temperature monitors and using a packing configuration designed to ensure an acceptable temperature range (1-10°C) was maintained. All were delivered within 24 hours of being packed. Units were stored in a monitored 1 to 6°C refrigerator and were sampled twice: once early in storage (3-7 days post collection) and once at the end of storage (40-43 days

[i] QMP testing conceived and performed by Canadian Blood Service research staff in Edmonton, AB and Ottawa, ON; Dr. Jason Acker, Tracey Turner, Adele Hansen, Jayme Tchir, Ioana Croteau, Craig Jenkins, Ken Wong and Jarret Webster.

[ii] WB collection and component processing performed by Canadian Blood Services staff.

post collection; expiry is Day 42). Units were gently massaged and inverted five times, and a 6 to 20-mL sample was drawn through a sampling site coupler. (4C2405, Fenwal) using an 18-gauge needle attached to a 10- to 25-mL syringe.

Processed RCCs were shipped under refrigerated conditions (1-10°C) to Canadian Blood Services in Edmonton, Alberta, Canada, within 24 hours of WB processing. Product testing for the Canadian Blood Services QMP was performed by members of Dr. Acker's lab as previously described[10]. Units were stored at 1-6°C and sampled twice: once 3-7 days post-collection, and again at the end of storage (40-43 days post-collection). Prior to sampling, RCCs were mixed by gentle kneading and inversion. 6-20 mL of sample was drawn through a sampling site coupler (4C2405, Fenwal) using an 18-gauge needle attached to a 10-25 mL Luer lock syringe.

An RBC sample was drawn into a capillary tube and centrifuged for 5 minutes at 14,850 × g at room temperature (Haematokrit 2010, Andreas Hettich GmbH & Co., Tuttlingen, Germany). Hematocrit (Hct) was determined visually. Total unit hemoglobin concentration was determined spectrophotometrically, using RBCs diluted (1:200) in Drabkin's reagent (0.61 mmol/L potassium ferricyanide, 0.77 mmol/L potassium cyanide, 1.03 mmol/L potassium dihydrogen) as previously described (Chapter 3 – 3.2.2). Of the units tested for QMP, 74 fell within the scope of this investigation; units from male and female donors aged 18-40 or ≥60.

5.2.2. Storage Study

WB was collected from 6 donors from each donor group (females aged 18-40, females aged ≥60, males aged 18-40, males aged ≥60) into Fenwal BC collection sets (CGR8441B, Quad PackPure

WB 500 mL, Fenwal), and processed using the BC processing method according to Canadian Blood Service's SOPs, as previously described (Chapter 2 – 2.2.1, Figure 2.1)[10][i]. RCCs were stored at 1-6°C and sampled at day 3, 21 and 43 following WB processing. Prior to sampling, RCCs were mixed by gentle kneading and inversion. 2 mL of sample was drawn through a sampling site coupler (4C2405, Fenwal) using an 18-gauge needle attached to a 10 mL Luer lock syringe[ii].

5.2.3. Measuring RBC indices

For both the QMP testing protocol and the storage study; MCV, MCH and MCHC were determined using a haematology analyser (Coulter AcT 8, Beckman Coulter Inc.), as previously described (Chapter 3 – 3.2.2).

5.2.4. Assessing RBC density

RBC density was assessed in fresh whole blood samples at day 3 following collection, and in stored RCCs at day 3, 21 and 43 following WB processing as a potential indicator of cell senescence. The ratio of high to low density RBCs was determined visually by separating the cells through a known density gradient[iii]. A density reference media (Percoll, Sigma Aldrich) consisting of colloidal silica beads coated in polyvinylpyrrolidone with a density of 1.130 g/mL was diluted in distilled de-ionized water to create two working density solutions with a density of 1.105 g/mL (solution A) and 1.115 g/mL (solution B) respectively. Due to the volume occupied by the solid silica particles, these electrolytes in solution will have an increased effective solution, causing the percoll

[i] WB collection and component processing performed by netCAD staff at the Centre for Blood Research, University of British Columbia, Vancouver, BC.
[ii] Sample preparation and RBC indices measurements performed by research staff in Dr. Jason Acker's lab; Tracey Turner, Anita Howell, April Xu and Angela Hill.
[iii] Protocol for assessing RBC density designed by Dr. Jason Acker, Anita Howell and I. RBC density assessment data collection performed by myself.

solutions to by hyperosmolal. Prior to RBC density separation, the osmolality of each RBC sample was measured using an osmometer, and separate working samples of Percoll solution A and B were drawn off and diluted with 1.5 M NaCl to correct their osmolality to that of the RBC sample using the following formula:

$$V_p = V_c \times [(O_c - O_f) / (R \times \{O_f - O_p\})]$$

Where; V_p = number of parts of Percoll solution, V_c = number of parts 1.5 M NaCl, O_c = osmolality of 1.5 M NaCl (2880 mOsm), O_f = desired osmolality (that of RBC sample), R = ratio of aqueous volume to total volume of Percoll, O_p = osmolality of uncorrected Percoll working solution.

To assess RBC density for each sample, 50 µL of Percoll solution A was drawn into a capillary tube followed by 50 µL of Percoll solution B, 25 µL of RBC sample was finally drawn into the same tube which was then sealed on the bottom (Figure 5.1). Prepared capillary tubes were centrifuged at 2200 x g for 10 minutes allowing the RBCs to fractionate between the layers of Percoll. Separation of density fractions was then assessed visually; the length of each fraction within the capillary tube was measured and the ratio of the different fractions was calculated from these measurements.

5.2.5. Statistical analysis

Statistical analysis was completed using statistical analysis software (SAS 9.1, SAS, Cary, NC. and SPSS 21, IBM, Armonk, NY)[i]. Mixed model analysis was performed to evaluate statistical significance of each pre- and post-storage RBC characteristics. For each output variable, a

[i] Statistical analysis performed by myself.

univariate, random effects mixed model was fitted, including just one predictor and using unique donor ID as the random effects variable to account for the potential clustering effect of repeat donor populations. Other variables were then added one at a time to assess the adjusted effect of the initial predictor with the confounding effects of other pre- and post-storage variables. A p-value of less than 0.05 was considered significant for both databases.

5.3. Results

5.3.1. Donor sex and age influence pre-storage RBC characteristics in leukoreduced and non-leukoreduced RCCs

Unit haematocrit was found to be lower in units from female donors than male donors in both pre-storage non-LR WB and pre-storage LR-RCC samples (Table 5.1, $p < 0.05$). Additionally, non-LR WB and LR-RCCs from young female donors (aged 18-30) had significantly lower unit haematocrit than units from females over 60, whilst the reverse trend was observed in units from male donors, with increasing age correlating with decreasing haematocrit (Table 5.1). Mean pre-storage unit MCV was also found to be influenced by donor sex; female donors exhibited overall increased MCV values than male counterparts, whilst the influence of donor age was again different for male and female populations. Young female donors were shown to have increased MCVs than older females, whilst young male donors exhibited decreased MCV values compared to older males (Table 5.1). Pre-storage MCHC values were not significantly different between donor groups in LR-RCCs, but MCHC differences between the donor groups were observed in non-LR WB. In non-LR WBs, female donors exhibited increased MCHC values compared to male donors; with increasing donor age being associated with decreased MCHC in both sexes (Table 5.1).

5.3.2. Component production and leukoreduction of RCCs influences pre-storage RBC characteristics

Due to the different additives and diluents present in the LR-RCC samples compared to the non-LR WB samples, Hct values were higher in all LR samples. Component processing and lukoreduction was also found to influence pre-storage MCV, MCHC and mean cell density ratio in processed RCCs (Table 5.1, Figure 5.1). Mean cell volume was increased in LR-RCCs compared to non-LR WB in all donor groups, whilst MCHC values were lower in LR-RCCs compared to non-LR WB in all donor groups (Table 5.1, LR-RCC: MCV = 93.5 ± 4.3 fL, MCHC = 336 ± 5.7 g/L, non-LR WB: MCV = 91.8 ± 4.0 fL, MCHC = 345 ± 4.9 g/L, $p < 0.05$). LR-RCCs were found to have a much higher fraction of low density RBCs when compared to non-LR WB across all donor groups (Table 5.1, Figure 5.1, LR-RCCs: 2.00 ± 0.73, non-LR WB: 0.66 ± 0.40, $p < 0.05$).

5.3.3. Donor sex and age influence rate of RBC density increase associated with RCC storage

Mean unit RBC density was found to increase throughout storage at a rate dependent on donor sex and age (Figure 5.2). Female RBCs were found to have lower density than male RBCs prior to storage (Table 5.1, Figure 5.1), and female RCCs exhibited a greater increase in mean unit RBC density than RCCs from male donors. Following 42 days of hypothermic storage, the mean RBC densities were much more comparable between donor groups than in pre-storage RCCs. At day 43 there was a statistically significant difference in RBC densities between the young male donors and the young female donors (Figure 5.2, young males: 0.82 ± 0.21, young females: 1.28 ± 0.34, $p < 0.05$), but there was no significant difference between other donor groups.

5.3.4. Donor sex and age influence RBC volume and haemoglobin content throughout RCC storage

Unit MCV was found to increase throughout storage at a rate dependent on donor sex and age, whilst MCHC was found to decrease throughout storage, also as a function of donor sex and age (Table 5.2, Figures 5.3 & 5.4). Older male donors exhibited the greatest rate of MCV increase throughout storage, whilst older females exhibited the slowest rate of MCV increase (Table 5.2, Figure 5.3, ΔMCV (day 3 – day 43): F 18-40 = 3.5 fL, F >60 = 3.1 fL, M 18-40 = 3.2 fL, M >60 = 3.9 fL). Older females also exhibited the slowest rate of MCHC decrease throughout storage, whilst young females exhibited the greatest rate of change (Table 5.2, Figure 5.4, Δ MCHC (day 3 – day 43): F 18-40 = 24 g/L, F >60 = 5 g/L, M 18-40 = 3.2 g/L, M >60 = 3.9 g/L). Unit MCH and mean unit haematocrit were not significantly influenced by storage duration in any of the donor groups.

5.4. Discussion

The aim of this study was to assess characteristics of RBC turnover and cell senescence (cell density, MCV, cell haemoglobin content) in different donor groups, as a potential mechanism in the observed effects of donor sex and age on storage haemolysis. Fresh non-leukoreduced whole blood samples were analysed, as well as both fresh and stored LR-RCCs, in order to assess the effects of processing and storage on cell senescence in different donor groups. In non-leukoreduced whole blood we observed lower density, larger RBCs in female donors compared to male donors, whilst donor age was found to have a sex dependent effect on cell volume. Component processing and leukoreduction of RCCs was found to increase MCV and decrease cell density in all donor groups. The influence of storage duration on RBC density, MCV and MCH in stored RCCs was shown to be dependent on donor sex and age.

Donor factors such as age, sex and ethnicity have been reported to influence both in vitro RCC quality characteristics; notably, RCCs from young female donors have been associated with the least amount of storage induced haemolysis[1, 2]. Several potential mechanisms for these observations have been hypothesised, based on known physiological differences between donor sub-populations. The sex hormones testosterone and progesterone have reported effects on RBC osmotic fragility, and have been suggested to play a role in the influence of donor age and sex on haemolytic propensity. It has also been suggested that regular blood loss in pre-menopausal female donors results in a less senescent population of circulating RBCs[5], which may be a factor in RBC osmotic fragility and susceptibility to damage and lysis during storage.

I*n vivo* RBC senescence is not fully understood, and whilst a number of morphological and biochemical processes have been ascribed to the cell aging process, the reports are inconclusive. Changes in cell shape resulting in loss of cell volume and surface area, increased cell density and reduced deformability have been reported in senescent RBCs[7-9]. In this study we observed high MCVs and lower cell densities in female blood compared to male blood. Young female donors were found to have the largest, least dense RBCs prior to storage; this may suggest that young female donors have a younger average population of circulating RBCs than other donor groups. Mature RBCs have also been reported to lose haemoglobin as they age[11], however we did not observe a significant effect on MCH based on donor age or sex. A proposed mechanism for the loss of cell volume, membrane surface area and haemoglobin content is the constant release of microvesicles by the RBC throughout its lifecycle[12].

In vitro RBC senescence is thought to be quite different to *in vivo* aging, with factors such as greatly reduced temperatures, reduced availability of metabolites and limited waste buffer capacity influencing the aging process. In this study we found that WB component processing and leukoreduction resulted in a lower mean cell density/increased cell volume. It is possible that the leukoreduction process effectively removes a population of high density cells. A key factor in RBC senescence is the externalisation of markers (primarily phosphatidylserine), resulting the recognition by the mononuclear phagocyte system and subsequent phagocytosis[7, 11]. Externalisation of phosphatidylserine is associated with increased cell adhesion; one could speculate that senescent RBCs exposing adhesive markers on their surface, adhere to the filter during leukoreduction. Storage of RBCs was associated with increasing cell density, which fits the model of *in vivo* RBC senescence. However, increasing RCC storage duration was also associated with increasing MCV; the reverse trend that one would expect when cells are increasing in density.

The observation that donor sex and age influence RBC cell density and MCV, and that young female donors have the largest, least dense RBCs prior to storage, supports the theory that menstrual blood loss in pre-menopausal women results in younger circulating cells. Further investigation into the how RBC age affects storage haemolysis and transfusion outcomes is critical in order to optimise transfusion processes to maximise patient safety and product quality and availability.

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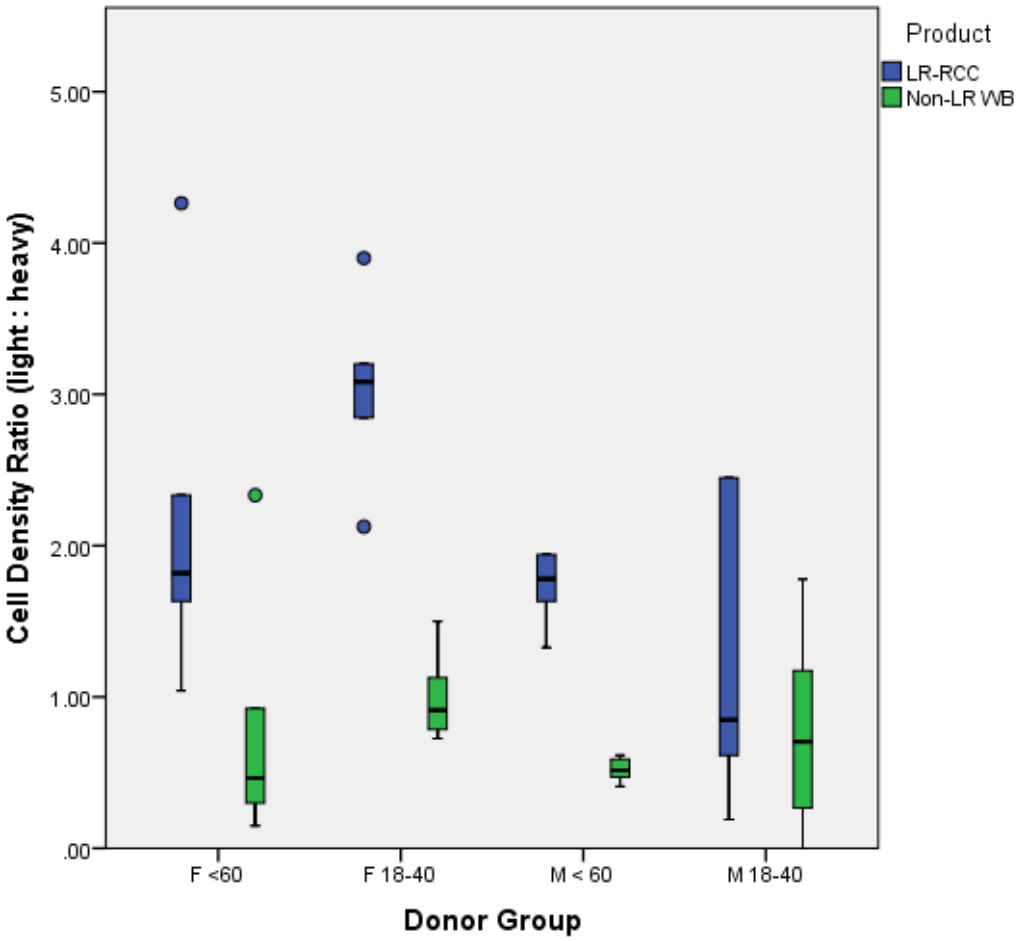
5.6. Tables & Figures

Table 5.1: Donor sex and age, RCC component processing and leukoreduction influence RBC characteristics in pre-storage RCCs

	Non-leukoreduced Whole Blood				Leukoreduced Red Cell Concentrate			
Donor Group	Hct (%)	MCV (fL)	MCHC (g/L)	Cell density ratio (light : heavy)	Hct (%)	MCV (fL)	MCHC (g/L)	Cell density ratio (light : heavy)
Females 18-40 (n = 6)	39.8 (± 2.5)	93.7 (± 3.3)	349 (± 4.8)	0.96 (± 0.30)	57.0 (± 2.6)	96.7 (± 2.6)	335 (± 7.4)	3.00 (± 0.58)
Females ≥60 (n = 6)	41.3 (± 0.6)	91.5 (± 4.2)	348 (± 8.0)	0.59 (± 0.81)	57.5 (± 1.5)	93.9 (± 4.8)	334 (± 6.5)	1.49 (± 1.12)
Males 18-40 (n = 6)	46.2 (± 1.6)	88.1 (± 5.7)	339 (± 4.3)	0.56 (± 0.69)	61.0 (± 1.5)	88.7 (± 5.6)	337 (± 5.1)	1.39 (± 0.98)
Males ≥60 (n = 6)	44.0 (± 1.6)	93.8 (± 2.8)	342 (± 2.8)	0.52 (± 0.07)	60.0 (± 3.3)	94.7 (± 4.3)	337 (± 3.6)	2.13 (± 0.24)

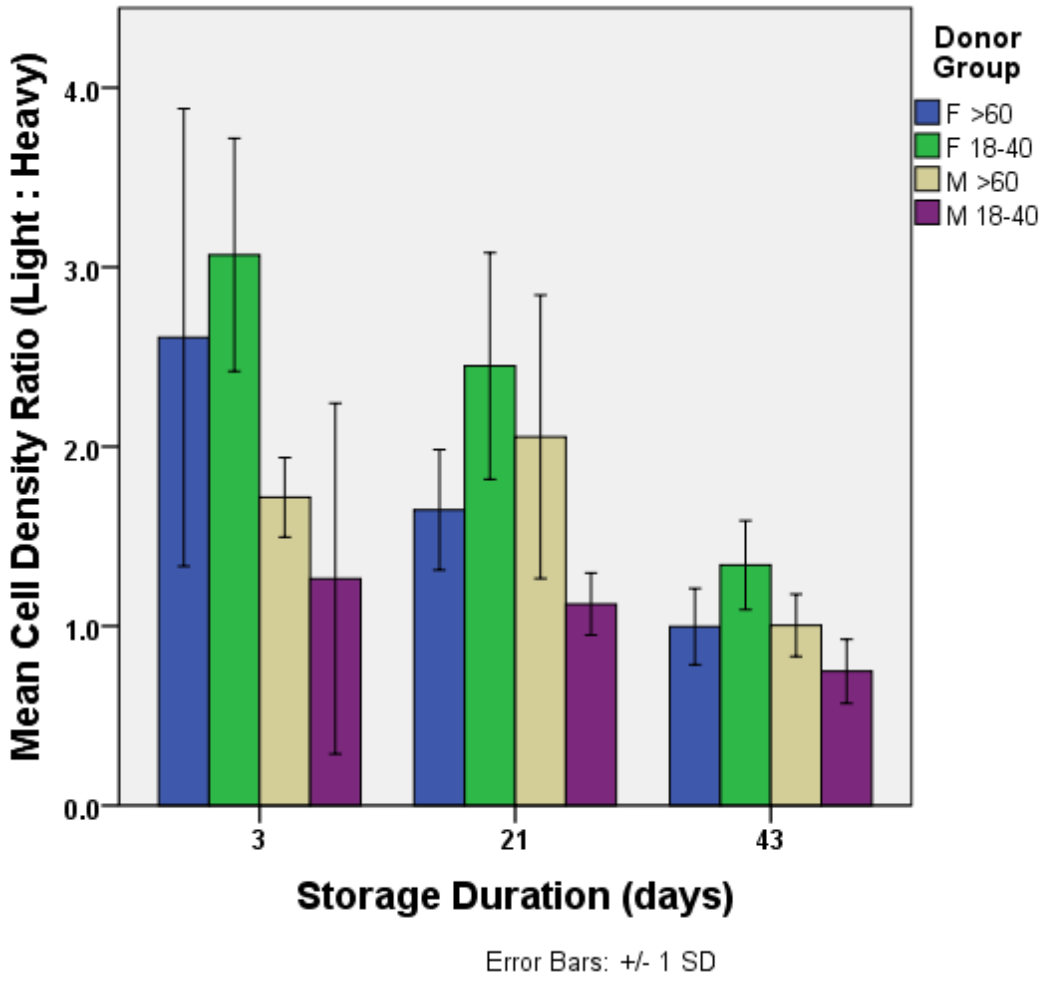
Measurements for haematocrit (Hct), mean cell volume (MCV), mean cell haemoglobin concentration (MCHC) and the ratio of light cells to heavy cells based on percoll separation, obtained on day 3 following collection. Total sample size of 24, with 6 donors from each group. Non-leukoreduced whole blood transported and stored in EDTA anticoagulant at 2-6°C prior to testing. Leukoreduced RCCs processed using "buffy-coat" processing method, transported and stored in SAGM additive solution at 2-6°C prior to testing.

Figure 5.1: Component processing and leukoreduction of RCCs removes a population of high density RBCs



Box plot represents the mean ratio of low density cells to high density cells in LR-RCCs and non-LR WB from each donor group, markers represent outlying data points. Measurements obtained on day 3 following collection. Total sample size of 24; 6 donors in each group, 12 LR-RCC, 12 non-LR WB. Non-LR WB transported and stored in EDTA anticoagulant at 2-6°C prior to testing. LR-RCCs processed using "buffy-coat" processing method, transported and stored in SAGM additive solution at 2-6°C prior to testing.

Figure 5.2: RBC density increases throughout storage and is dependent on donor sex and age



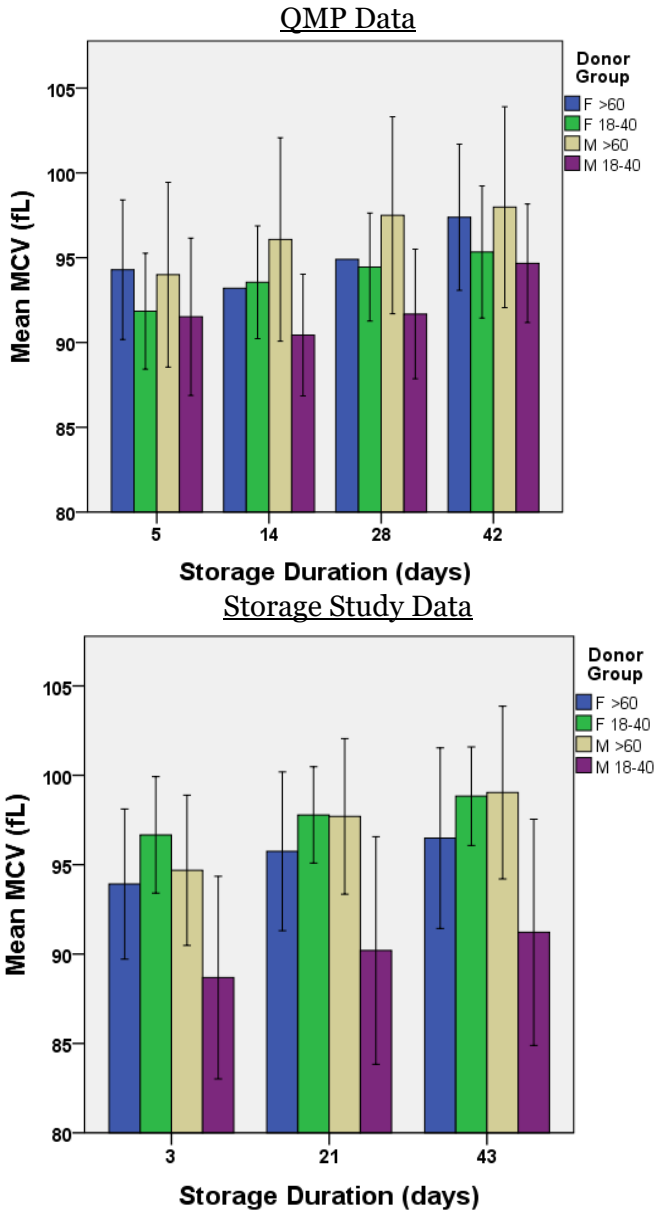
Mean ratio of low density cells to high density cells as a function of storage duration and donor sex and age. RBCs stored at 2-6°C in SAGM additive solution. RBC density measured using Percoll separation on day 3, 21 and 43 following collection. Total sample size of 24, with 6 donors in each donor group.

Table 5.2: Mean cell volume, haemoglobin concentration and unit haematocrit are dependent on donor sex and age in stored RCCs

	Fresh				Expiry			
Donor Group	Hct (%)	MCV (fL)	MCH	MCHC (g/L)	Hct (%)	MCV (fL)	MCH	MCHC (g/L)
Females 18-40 (n = 12)	59.1 (± 2.3)	91.8 (± 3.3)	29.7 (± 1.4)	323 (± 10.4)	60.0 (± 3.0)	95.3 (± 3.7)	28.6 (± 1.7)	299 (± 8.4)
Females ≥60 (n = 14)	59.8 (± 1.9)	94.3 (± 4.0)	29.2 (± 1.5)	310 (± 13.1)	59.4 (± 2.1)	97.4 (± 4.2)	29.7 (± 1.5)	305 (± 8.7)
Males 18-40 (n = 24)	62.4 (± 2.5)	91.5 (± 4.5)	29.6 (± 1.9)	324 (± 15.0)	61.5 (± 3.1)	94.7 (± 3.4)	28.9 (± 1.7)	305 (± 11.5)
Males ≥60 (n = 24)	61.0 (± 1.7)	94.0 (± 5.3)	30.4 (± 2.2)	323 (± 14.1)	60.3 (± 1.3)	97.9 (± 5.8)	30.0 (± 2.1)	307 (± 9.0)

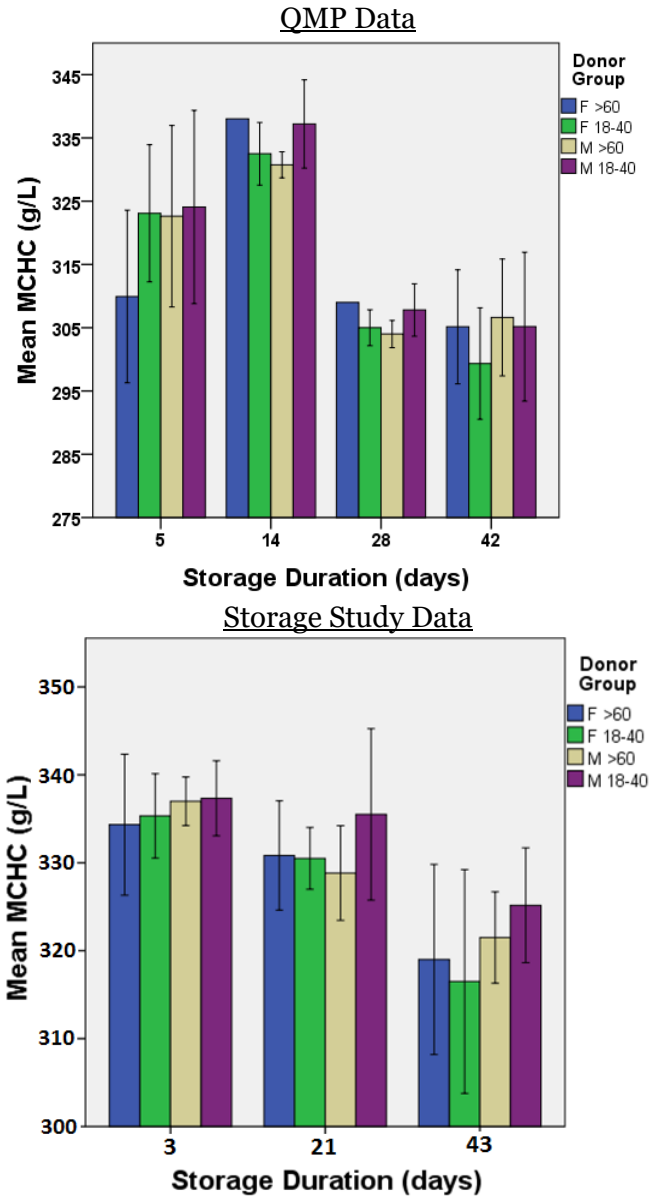
QMP measurements for haematocrit (Hct), mean cell volume (MCV), mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) obtained on day 5 following collection. N = 74. Leukoreduced RCCs processed using “buffy-coat” processing method, transported and stored in SAGM additive solution at 2-6°C prior to testing.

Figure 5.3: Mean cell volume increases during RBC storage and is dependent on donor sex and age



Mean cell volume (MCV) as a function of storage duration and donor sex and age for stored RCCs from the Canadian Blood Services QMP (top graph, n = 74) and from the RCC population from the RBC density storage study (bottom graph, n = 24). RBCs were stored at 2-6°C in SAGM additive solution, MCV was measured using a haematology analyser on day. Testing was performed on days 5, 14, 28 and 42 following collection (QMP), and 3, 21 and 43 days following collection (storage study data). Bars represent mean MCV for each donor group at each testing point, error bars represent ±1 standard deviation.

Figure 5.4: Mean cell haemoglobin concentration decreases during RBC storage and is dependent on donor sex and age



Mean cell haemoglobin concentration (MCHC) as a function of storage duration and donor sex and age for stored RCCs from the Canadian Blood Services QMP (top graph, n = 74) and from the RCC population from the RBC density storage study (bottom graph, n = 24). RBCs were stored at 2-6°C in SAGM additive solution, MCHC was measured using a haematology analyser on day. Testing was performed on days 5, 14, 28 and 42 following collection (QMP), and 3, 21 and 43 days following collection (storage study data). Bars represent mean MCHC for each donor group at each testing point, error bars represent ±1 standard deviation.

Chapter 6

Conclusions

6.1. Summary of Key Findings

The preservation and storage of human blood components for transfusion is a vital part of modern health care systems; allowing blood services to meet the growing demand for safe, effective transfusion components. Advancements in RBC storage additive solutions and hypothermic preservation processes have allowed for increased storage durations. Recently, however, there has been a great deal of debate regarding the safety of stored RCCs and the potential clinical implications of long term RBC storage. Initial studies to assess the relationship between RCC storage duration and transfusion outcomes were inconclusive, and it has since been recognised that sources of variability in stored RCCs extend beyond just storage conditions. The aim of this thesis was to investigate potential pre-storage sources of *in vitro* variability in stored RCCs; focussing on donor characteristics and whole blood component processing conditions.

The aim of chapter 2 was to assess the influence of component separation technique (whole blood filtered processing vs. buffy-coat processing) and donor sex and age on RCC storage haemolysis. We observed that BC-RCCs exhibited lower levels of storage haemolysis than WBF-RCCs. We also observed lower levels of storage haemolysis in RCC from female donors compared to male donors, and reported a correlation between donor age and storage haemolysis. Based on these

observations, we sought to investigate the mechanisms by which these pre-storage factors influence the rate of storage haemolysis in RCCs. In order to investigate the reason for the increased storage haemolysis we observed in WBF-RCCs, an experiment was performed to measure the volume of additive solution and residual plasma present in stored WBF-RCCs compared to stored BC-RCCs. The aim of chapter 3 was to assess the influence of residual plasma volume and additive solution volume on RCC storage haemolysis, as a potential explanation for the observed effects of processing conditions on RCC storage haemolysis. We observed that within the scope of blood banking conditions, residual plasma did not have a significant effect on RCC storage haemolysis. Only at extreme ratios did additive solution and residual plasma have an effect on the rate of storage haemolysis.

The aim of chapter 4 was to further investigate the role of donor sex and age in RCC storage haemolysis; assessing how donor sex and age influence donor haemoglobin levels, and how these donor characteristics are associated with RCC haemolysis, unit haematocrit and unit haemoglobin content. We observed that pre-donation donor haemoglobin levels were a function of donor sex and age; young female donors had the lowest mean pre-donation haemoglobin levels, whilst young males had the highest. Donor sex, age and pre-donation haemoglobin all had a direct effect on RCC haematocrit and haemoglobin content; however, the effect of donor sex and age on RCC characteristics was found to be independent of donor haemoglobin levels. High frequency of donation was found to be associated with decreased pre-donation haemoglobin levels, with young male donors showing the sharpest drop in haemoglobin level. Donation frequency was also shown to influence unit haemoglobin, unit haematocrit and unit haemolysis in a manner dependent on donor sex and age.

The aim of chapter 5 was to investigate the relationship between donor sex and age, and the size, density and haemoglobin content of circulating RBCs. As decreasing cell size and haemoglobin

content have been associated with *in vivo* RBC senescence, this study was designed to assess pre-donation RBC senescence in different donor groups as a potential mechanism behind donor dependent storage haemolysis in RCCs. We observed that mean cell volume and density were both dependent on donor age and sex. In fresh cells; young female donors exhibited the largest, least dense RBCs of all donor groups, with increasing donor age in the female population correlating with increased density and decreased size. In the male population, we observed the reverse trend; young male donors exhibited the smallest, highest density RBCs of all the donor groups, whilst increasing donor age was associated with decreasing cell size and density. Differences in MCHC were observed between donor groups, however MCH values were not statistically different between groups due to the observed differences in MCV. We also observed that leukoreduction and RCC component production has a significant influence on the MCVs and cell densities measured for each donor group. Storage was found to influence MCV and cell density at a rate dependent on donor sex and age, whereas storage did not seem to have a significant influence on MCH.

6.2. Significance of Key Findings

In trying to understand the clinical implications of the long term storage of RBCs for transfusion, much of the focus has been on storage duration. As discussed throughout this thesis, there is a great deal of potentially significant variability within the categories of "fresh" and "old" RCCs. We have demonstrated how the *in vitro* characteristics of stored RCCs are dependent on pre-storage conditions as well as storage duration. WB component processing conditions and donor factors such as sex, age, frequency of donation were all found to influence post-storage RCC characteristics. Evidence is now showing that the influence of these *in vitro* RCC characteristics on clinical transfusion outcomes is dependent on characteristics of the recipient patient[1]. To

accurately assess the “quality” of stored RCCs, there are factors to take into account from donor, through processing and storage, up to the recipient.

The screening of donors is a key part of blood banking services, important for recruiting appropriate donors, limiting the collection of unsafe or otherwise unusable blood, and ensuring the safety of donor. As demonstrated in this thesis, there are a number of donor factors, or more accurately donor blood characteristics, that influence the quality characteristics of stored RCCs. The observations presented here and by other groups regarding the influence of donor characteristics on RBC storage, and also the observations by other groups regarding the influence of donor factors on clinical transfusion outcomes, could be managed using existing donor screening systems. By understanding how a unit of RCC collected from a patient with a certain set of characteristics will behave during storage, blood services will be able to further optimise the management of their supply of blood.

Blood component manufacturing processes and equipment are constantly being developed and optimised, with the aim of increasing production efficiency and product quality and safety. The data presented here shows how processing conditions influence quality characteristics in stored RCCs. A better understanding of how component processing can influence RCC storage will help guide process development towards producing better quality, safer transfusion components. We demonstrate interplay between manufacturing conditions and donor characteristics as determinants of RCC quality; through component manufacturing we are able to compliment certain donor blood characteristics, or mitigate unwanted characteristics.

6.3. Future Directions

The observations and analyses presented in this thesis give rise to a number of questions and issues that warrant further study. The mechanisms and physiological differences accounting for the observed role of manufacturing conditions and donor factors in product quality are still not fully understood. One of the key elements to fully understanding RCC quality is in developing a definitive model of the relationship between donor factors, manufacturing and storage conditions, recipient patient factors and clinical transfusion outcomes. This is likely a hugely multifactorial process, and an accurate model will need to incorporate all the appropriate variables. This is a massive undertaking, and would require an international effort to capture the broadest scope of potential variables; incorporating a wide donor demographic, and localised blood banking practises. In order to be sure of the clinical implications of the observed variability within RCC products, a more extensive investigation is necessary, linking unit characteristics (pre-transfusion sources of variability such as donor factors, storage duration, etc.) and recipient patient characteristics, with transfusion outcomes.

The observation that RBC size and density in fresh and stored RCCs are a function of donor sex and age raises questions about the process of *in vivo* RBC senescence in different donor populations. Further research is necessary to better define the biochemical processes of *in vivo* RBC senescence, and how these processes differ from *in vitro* cell aging during hypothermic storage. Further study is also necessary to determine how the processes of *in vivo* RBC senescence differ in different donor populations. Assessing a broad array donor factors to include specific genetic traits or phenotypes would help to better understand the scope of donor variability. Building upon our observations the MCV and cell density are influenced by donor sex and age, future studies could focus on the other aspects of the current model of cell senescence, for example; microvesiculation, loss of membrane asymmetry/phosphatidylserine exposure and

changes in cell shape. Further research is also necessary to determine the influence of pre-donation RBC senescence on *in vitro* cell aging during storage. By accurately defining, and then isolating populations of RBCs of different cellular ages, one could expose these cells to storage conditions and monitor the RBC characteristics.

The observations presented in this thesis regarding the influence of donation frequency on donor RBC characteristics warrant further investigation. Blood loss through regular donation may influence RCC storage in a similar manner to menstrual blood loss in pre-menopausal female donors. Understanding one of these phenomenon is likely to aid in understanding the other. Future directions for research in this area could include looking at the average interval between donations as well as the number of donations within a set time period, and expanding the number of RBC parameters measured.

In this thesis I have presented a broad look at how the quality of stored RCCs is dependent on the characteristics of the donor's blood and the conditions of component processing, as well as duration of storage. Through analysis of the information presented here and by other groups, and through speculation, we can begin to build a picture of the various processes affecting the quality and safety of stored RCCs. However, the mechanisms and the biochemistry driving these processes are yet to be fully understood. In its current state, the global blood banking industry provides safe, effective transfusion components to millions of patients around the world, and undoubtedly saves a huge number of lives. In the continuous strive for improvement, further development of transfusion medicine needs to be guided by a solid understanding of the biological processes that govern product safety and efficiency.

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Appendix A

Determining the volume of additive solution and residual plasma in whole blood filtered and buffy-coat processed red cell concentrates[i]

A.1. Introduction

Following collection, whole blood is processed into transfusable components, which are then stored until required. During WB processing, RBCs are separated from plasma and other WB components by centrifugation. Blood services utilize a number of different processing techniques and use different centrifugation settings to fractionate transfusable components. Using currently available techniques, it is not possible to ensure the complete separation of plasma from the RBCs. The volume of residual plasma remains with the RBC component throughout storage and the potential impact this has on RBC quality and transfusion outcome has not been fully investigated. Previously, the volume of residual plasma in stored red cell concentrates has been calculated using the following equations [1]:

[i] A version of this appendix has been published under this title in Transfusion Medicine and Hemotherapy, 2016, vol. 43, pg. 133-136.

Residual plasma volume (mL) = Supernatant volume (mL) - Additive solution volume (mL) Eq 1

Supernatant volume (mL) = Total Volume (mL) x (1 - hematocrit) *Eq 2*

RCC components at Canadian Blood Services are stored in saline-adenine-glucose-mannitol (SAGM) additive solution. The additive solution volume in the collection sets is approximately 110 mL. This is a nominal value for the additive solution as the suppliers give a margin of error of 104.5 mL – 121 mL. We previously attempted to calculate the volume of residual plasma in Canadian Blood Services RCC products by applying Equations 1 and 2 to a large quality control database [2]. Using an expected additive solution volume of 110 mL, we observed a high frequency of negative values for residual plasma volume, and significantly lower values in components processed using the buffy-coat (BC) method. This led us to speculate that less than 110 mL of additive solution is delivered to the RBCs during processing, and that this value is affected by processing method. To investigate this further, we set out to accurately measure the volume of SAGM additive solution and residual plasma in WB filtration (WBF), and BC-processed RCC components.

A.2. Materials & Methods

A.2.1. Whole Blood Collection and Processing

WB was collected from 3 donors using Macopharma WBF collection sets (DQE 7291 LX Leucoflex MTL1 Quadruple Top and Top System, CPD/SAGM 500 mL, MacoPharma, Tourcoing, France), and from 3 donors using Macopharma BC collection sets (LQT 7291 LX leucoflex LCR-Diamond Quadruple Bottom and Top System, CPD/SAGM 500 mL), and processed according to Canadian Blood Services standard operating procedures, as previously described (Chapter 2 – 2.2.1,

Figure2.1)[3][i]. Briefly; WBF units are leukoreduced then centrifuged at 4552 x g for 6 minutes to produce one unit of RCC and a unit of plasma. BC units are centrifuged prior to leukoreduction at 3493 x g for 11 minutes, yielding a unit of buffy coat in addition to the plasma and RCC. At given points throughout processing, samples were taken for mannitol and albumin measurements (Figure A.1).

A.2.2. Component Characteristics

Total unit volume was calculated by dividing the tare weight (g) of the component by 1.06 g/L (specific gravity of WB at a standard hematocrit). Haematocrit was determined using a Coulter haematology analyzer (Coulter AcT 8 Hematology Analyzer, Beckman Coulter, Brea, CA, USA), which calculates haematocrit from MCV and cell count.

A.2.3. Mannitol Measurements

Mannitol concentration was measured using a colorimetric assay (D-Mannitol Colorimetric Assay Kit, Sigma-Aldrich, St. Louis, MO, USA)[ii]. For both WBF and BC processed units, a sample of SAGM was taken from the SAGM container before mixing with the RBCs to provide a baseline SAGM mannitol concentration. For WBF processed units, samples were taken from the RCC container after the SAGM had been thoroughly mixed with the RBCs. For BC processed units, samples were taken from the RCC storage container after the SAGM had been mixed with the RBCs and the SAGM-RBC mixture had passed through the leukoreduction filter. RCC samples were centrifuged at 2200 x g for 10 minutes to separate the RBCs and supernatant. Twenty-five

[i] WB collection and component processing performed by netCAD staff at the Centre for Blood Research, University of British Columbia, Vancouver, BC.
[ii] I performed mannitol concentration assays.

µL of supernatant was removed and diluted in an equal volume of mannitol assay buffer (MAK096A, Sigma Aldrich). Samples and standards were added to reaction mixtures (46 µL mannitol assay buffer, 2 µL mannitol enzyme mix, 2 µL mannitol substrate mix) in a 96 well plate and incubated for 20 minutes at 37°C. The assay is a coupled enzyme assay; enzymes in the reaction mixture bind to mannitol and form a compound that can be measured colorimetrically at 450 nm using a spectrophotometric plate reader (Spectramax 384+, Molecular Devices, Sunnyvale, CA, USA). The concentration of mannitol in each sample was determined against a standard curve of known concentrations (Mannitol Standard, MAK096D, Sigma Aldrich). The concentration of mannitol in the RCC was used to calculate the volume of SAGM that had been delivered using the following equation in which M_1 = RCC mannitol concentration (g/mL), V_1 = RCC volume (mL), M_2 = SAGM mannitol concentration (g/mL), V_2 = SAGM volume (mL):

$$V_2 = (M_1 \times V_1)/M_2 \qquad \text{Eq 3}$$

A.2.4. Albumin Measurements

Albumin was measured in the samples using a bromocresol green colorimetric assay (BCG Albumin Assay Kit, Sigma-Aldrich)[i]. For both WBF and BC processed units, samples were taken from the final leukoreduced SAGM-RCC component, and also from the separated plasma component for measuring plasma albumin concentration. Plasma samples were diluted 2-fold with water, RCC samples were centrifuged at 2200 x g for 10 minutes to separate the RBCs and supernatant and the supernatant was removed. Samples and standards were added to BCG albumin assay reagent (Sigma Aldrich) in a 96 well plate and incubated at room temperature for 5 minutes. The bromocresol green reagent binds selectively with albumin and forms a green

[i] I performed all albumin measurements.

compound. Absorbance was read at 620 nm using a spectrophotometric plate reader (Spectramax 384+). The concentration of albumin in each sample was determined using a standard curve of known concentrations (BCG albumin assay albumin standard, 5 g/dL, Sigma Aldrich). The concentration of albumin was used to calculate the volume of plasma in the RCC using the following equation in which A_1 = RCC albumin concentration (g/mL), V_1 = RCC volume (mL), A_2 = plasma albumin concentration (g/mL) V_2 = residual plasma volume (mL):

$$V_2 = (A_1 \times V_1)/A_2 \qquad \text{Eq 4}$$

A.3. Results

Supernatant volumes calculated using total unit volume and hematocrit (Eq. 2) were significantly lower in BC processed units compared to WBF units ($p < 0.05$), and were less than 110 mL in all components excluding one WBF unit (112 mL; Table A.1). The volume of SAGM was calculated using mannitol concentrations taken from the SAGM (52.4 ± 0.1 g/µL) and RCC samples (BC = 16.4 ± 0.04 g/µL, WBF = 16.8 ± 0.03 g/µL; Eq. 3). Calculated SAGM volumes were greater in WBF processed RCCs compared to BC components ($p < 0.05$; Table A.2), which is consistent with the difference in supernatant volumes.

Using albumin concentrations from plasma components and processed RCC components (plasma: WBF = 4.43 ± 0.42 g/dL, BC = 4.32 ± 0.34 g/dL; RCCs: WBF = 0.22 ± 0.06 g/dL, BC = 0.19 ± 0.03 g/dL), we were able to calculate the volume of plasma remaining in RCCs (Eq. 4). Residual plasma volumes calculated using equation 4 were similar in both WBF and BC components ($p = 0.245$; Table A.2). A calculated residual plasma volume (Eq. 1) was obtained

using calculated supernatant volumes (Table A.1) and calculated SAGM volumes (Table A.2); this showed a non-significant difference between the volume of plasma in both types of unit ($p = 0.06$).

A.4. Discussion

Previously, the volume of residual plasma has been calculated using supernatant volume and the volume of additive solution [1,4]. When this calculation method was applied to a large scale QC database, supernatant values less than 110 mL were observed, returning negative values for residual plasma volume. In this study we sought to accurately calculate the volume of SAGM additive solution and residual plasma using mannitol and albumin concentrations.

We observed significantly lower supernatant volumes and additive solution volumes in BC components compared to WBF components. A key difference between the WBF and BC processing methods is the point of component leukoreduction (Figure A.1). In the BC process, filtration occurs after component separation. The SAGM is passed through the filter to "pre-wet" it before the SAGM-RBCs are filtered back through. The filter may act as a sink for additive solution during the pre-wetting step, accounting for the reduced volume of SAGM observed in these components. Previous studies have reported reduced storage hemolysis associated with larger volumes of additive solution, attributing increased buffer capacity as a possible reason [5].

Neither method used to calculate residual plasma showed a significant difference between WBF and BC-processed RCCs. However, this study is limited by its small sample size; a larger sample size may indicate a statistical significance. We did observe disparity between plasma volume calculation methods; values calculated using albumin concentrations were higher than the values calculated using equation 1. This disparity was only significant in WBF processed units ($p < 0.05$).

One possible explanation for this is that the dispersion of albumin, a large molecule, throughout the WB is affected by centrifugation.

The effect of residual storage plasma in RCCs has not been fully investigated, and its influence on product characteristics and transfusion outcomes is unknown. Transfusion of plasma is associated with TRALI [6], the leading cause of transfusion related deaths in North America. It has been suggested that larger volumes of residual plasma in RCCs could be associated with increased risk of TRALI [1, 7]. In addition to clinical outcomes, there is also interest in how residual plasma influences *in vitro* RCC quality during storage. In a recent study on neonatal RBCs stored in plasma, it was observed that high plasma lipid content was associated with increased storage hemolysis [8].

To be confident we can provide the safest possible products to patients, we must know exactly what is in the bag. The findings of this study emphasize that the supernatant in RCC products should not be viewed as a set volume of additive solution, but rather a variable mixture of plasma components and additive solution. It is important to understand sources of variability within our blood products, and to do this, standardized methods of measurement need to be in place.

A.5. References

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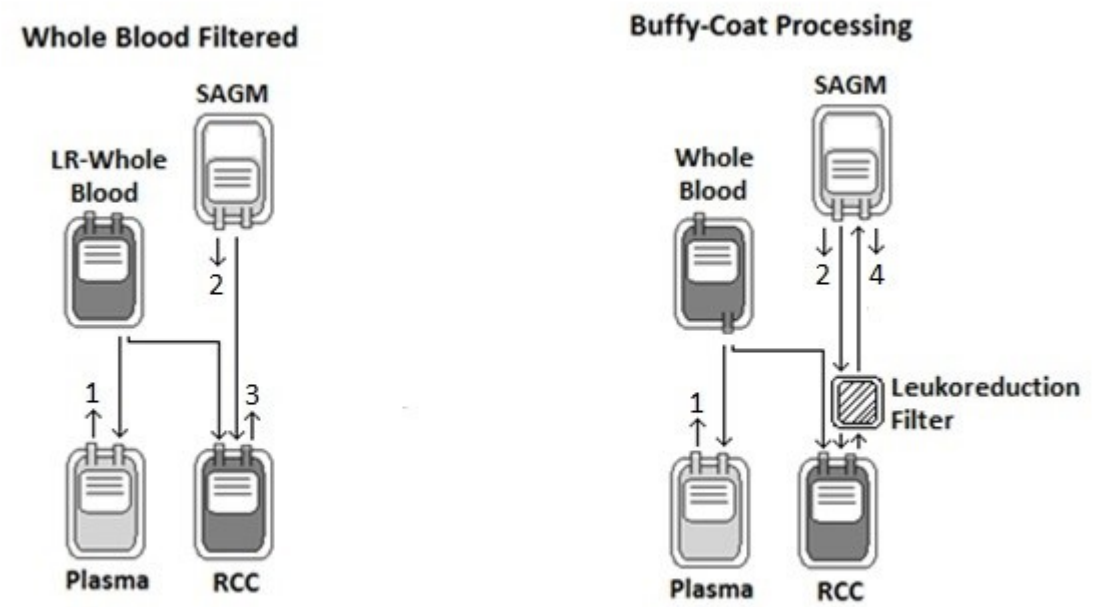
A.6. Tables and Figures

Figure A.1: Whole blood filtration and buffy-coat processing methods.



The sequence for sampling the WBF and BC RCCs for residual plasma and additive solution testing was as follows: 1) albumin samples were taken from plasma components; 2) mannitol samples were taken from the SAGM component prior its addition to the RBCs; 3) mannitol and albumin samples were taken from LR-RCC components following mixing with SAGM; 4) SAGM was drained through leukoreduction filter and mixed with RBCs, the SAGM-RBCs were passed back through filter into SAGM bag and mannitol and albumin samples were then taken from LR-RCC component following leukoreduction.

Table A.1: RCC unit volumes and hematocrits for WBF and BC processed components

	Total Unit Volume (mL)	**Hematocrit** (%)	**Supernatant Volume** (mL)
WBF (n = 3)	315 (±5)	66 (±1)	107 (±5)
BC (n = 3)	275 (±4)	65 (±1)	95 (±4)

Total unit volume was calculated by multiplying unit tare weight (g) by 1.06. Supernatant volumes were calculated using total unit volume and hematocrit (Eq. 2). Values shown are mean (±SD).

Table A.2: Mean volumes of SAGM and residual plasma in WBF and BC processed RCC components

	SAGM Volume (mL)	**Measured (A) Residual Plasma Volume** (mL)*	**Calculated (B) Residual Plasma Volume** (mL)†	**Residual Plasma** (%)	**Ratio of Plasma:SAGM**
WBF (n = 3)	101 (±2)	16 (±4)	6 (±3)	A: 4.7 (±1.4) B: 2.0 (±0.8)	A: 0.14 (±0.04) B: 0.06 (±0.02)
BC (n = 3)	86 (±3)	12 (±3)	9 (±2)	A: 3.9 (±1.1) B: 3.9 (±0.5)	A: 0.11 (±0.03) B: 0.12 (±0.01)

SAGM volumes were calculated from experimental mannitol concentrations (Eq. 3).

*Plasma volume was calculated from experimental albumin measurements (Eq. 4).

†Plasma volume was calculated using supernatant volume and SAGM volume (Eq. 1).

Values shown are mean (±SD).