The Effect of Washed vs Unwashed Packed Red Blood Cell Transfusion on Immune Responses in the Extremely Preterm Newborn:

A Randomised Trial

Tara Marie Crawford

School of Medicine/ Paediatrics

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“Anything that is not actually impossible can be done,

if one really sets one’s mind to do it and is determined that it shall be done”

- Sir Nicholas Winton

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Thesis Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Contributors Statement

A/Prof Michael Stark and Dr Chad Andersen conceptualised and designed the study. Ms Crawford coordinated the study, recruited participants, completed all data collection, conducted all laboratory analysis and undertook all data analysis.

Publications and Presented Abstracts

Publications

Crawford TM, Andersen CC, Stark MJ. Effect of repeat transfusion exposure on plasma cytokine and markers of endothelial activation in the extremely preterm neonate. Transfusion (in press) 26th May 2020

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Crawford TM, Andersen CC, Robertson SA, Hodyl NA, Stark MJ. Transfusion Related Circulatory Overload (TACO) Distinct from Acute Lung Injury (TRALI) in Very Preterm Newborns? 2020. In Press

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Abbreviations

2,3-DPG	Diphosphoglycerate
ANZNN	Australian and New Zealand Neonatal Network
AOP	Anaemia of Prematurity
ATP	Adenosine triphosphate
ARDS	Acute Respiratory Distress Syndrome
AV	Annexin V
BPD	Bronchopulmonary Dysplasia
CNS	Central Nervous System
CO	Cardiac Output
DBP	Diastolic Blood Pressure
ELBW	Extremely low Birth Weight
ELISA	enzyme-linked immunosorbent assay
EPO	Erythropoietin
FFP	Fresh Frozen Plasma
FiO2	Fraction of inspired oxygen
FMC	Flinders Medical Centre
GI	Gastrointestinal system
GPA	Glycophorin A
Hb	Haemoglobin
HbF	Fetal Haemoglobin
HLA	Human Leukocyte Antigen
HNA	Human Neutrophil Antigen
HR	Heart Rate
IFNγ	Interferon gamma

Ig	Immunoglobulin
IL	Interleukin
IUGR	Intrauterine Growth Restriction
IQR	Interquartile Range
IVH	Intraventricular Haemorrhage
LBW	Low birth weight
LVO	Left Ventricular Output
MAP	Mean Airway Pressure
MBP	Mean Blood Pressure
MCP	Monocyte Chemoattractant Protein
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
MIP	Macrophage Inhibitory Protein
MODS	Multiple Organ Dysfunction Syndrome
NEC	Necrotizing Enterocolitis
NICU	Neonatal Intensive Care Unit
NIRS	Near-infrared Spectroscopy
NK	Natural Killer Cell
nTACO	Neonatal Transfusion Associated Circulatory Overload
nTAD	Neonatal Transfusion Associated Dyspnea
NTBI	Non-Transferrin Bound Iron
nTRALI	Neonatal Transfusion Related Acute Lung Injury
PAI	Plasminogen Activator Inhibitor
PDA	Patent ductus arteriosus
PINT	Prematures In Need of Transfusion

PLTs	Platelets
PP	Pulse Pressure
PRBC	Packed Red Blood Cell
PS	Phosphatidylserine
PVL	Periventricular Haemorrhage
RBC	Red Blood Cell
RDS	Respiratory Distress Syndrome
Rh	Rhesus
ROP	Retinopathy of Prematurity
ROS	Reactive Oxygen Species
RR	Respiratory Rate
RSS	Respiratory Severity Score
RV	Right Ventricle
SAG-M	saline adenine glucose mannitol
SBP	Systolic Blood Pressure
SHOT	Serious Hazards of Transfusion
sICAM	Soluble Intercellular Adhesion Molecule
SIP	Spontaneous Intestinal Perforation
sVCAM	Soluble Vascular Cell Adhesion Molecule
SVR	Systemic Vascular Resistance
TACO	Transfusion Associated Circulatory Overload
TANEC	Transfusion Associated Necrotising Enterocolitis
Th	T Helper
TNF	Tumour Necrosis Factor
TRAGI	Transfusion Related Acute Gut Injury

TRALI	Transfusion Related Acute Lung Injury
TRIM	Transfusion Related Immunomodulation
VEGF	Vascular endothelial growth factor
WBC	White Blood Cell
WCH	Women's and Children's Hospital

Abstract

Packed red blood cell (PRBC) transfusions continue to result in adverse inflammatory responses and increased rates of morbidity despite modifications in processing such as leukodepletion. It remains unknown if this is due to adverse physiological responses, inflammatory processes related to transfusion related immunomodulation (TRIM) or both. Washing of PRBCs may reduce the immunomodulatory potential and transfusion related adverse outcomes. This study aimed to investigate whether transfusion with washed leukodepleted PRBCs in the preterm newborn reduces post-transfusion inflammatory cytokine responses compared to transfusion with unwashed leukodepleted PRBCs, improving physiological stability.

Extremely preterm newborns (n=154) were randomised to transfusion with unwashed or washed leukodepleted PRBCs, determined by the restrictive threshold of the PINT transfusion study, until primary hospital discharge (77 per arm). Plasma cytokines, markers of endothelial activation and measures of cardiorespiratory stability where measured pre- and post-transfusion.

Transfusion with washed PRBCs resulted in decreases in pro-inflammatory cytokines while unwashed PRBCs resulted in increases in IL-17A and TNF. By the 3rd transfusion this response to unwashed PRBCs was associated with increases in MIF and PAI-1, markers of endothelial activation, an effect not seen with washed PRBCs. This response was influenced by donor sex with exposure to PRBCs from a female donor resulting in increases in pro-inflammatory cytokines, an effect ameliorated by PRBC washing. Changes over time and in response to the type of packed red blood cells transfused were seen with a greater reduction in cardiac output and increase in systemic vascular resistance and mean airway pressure seen in those infants transfused with washed PRBCs. However, none of these alterations met the current accepted definitions for either transfusion-associated circulatory overload or transfusion-associated lung injury.

The current study suggests that the association between transfusion and poor outcome have a predominantly immunomodulatory basis, a relationship that may be altered by the use of washed packed red blood cells for the extremely preterm infant. The apparent lack of significant changes in cardio-respiratory responses to a transfusion may reflect the need for specific diagnostic criteria for transfusion related complication in the preterm newborn. The current results provide strong mechanistic data supporting a potentially beneficial effect of transfusion of washed packed red blood cells in this high-risk population. The next critical step is investigating whether the use of washed packed red blood cells is associated with a significant reduction in clinical outcomes, a finding which would have wide ranging implications for both transfusion medicine and the field of neonatology.

Chapter 1

Literature Review

1 Preterm Birth

1.1 Epidemiology of Prematurity

In Australia and New Zealand there are over 300,000 registered births each year. Of these, 3% (10,161) are born preterm (<37 weeks' gestation) or low birth weight (LBW) (<2500 grams) [1]. Despite substantial advances in perinatal management [2], preterm and/or LBW infants continue to have significant rates of both long term morbidity and mortality [3]. Further, over the past decade there has been a steady increase in the number of live births of extremely premature infants (<28 weeks' gestation) [1], with 8.7% of live births in 2017 classified as preterm (<37 weeks' gestation) [4]. It has been estimated that in Australia the incremental cost of each single extremely preterm infant reaching adulthood is $141,200 (AU) per annum with the estimated total cost of preterm birth $4.42 billion per annum [5]. As a result, this high-risk population imposes a significant economic burden to families and society, as well as the enormous stress and emotional toll. The economic burden is in part due to ongoing morbidity which persists beyond childhood and adolescence. In addition, infants born preterm in high-income countries are half as likely to achieve a higher education compared to their term counterparts and earn approximately 16% less as an adult, which is equivalent to a nearly 2% loss of total population earned income [6]. Therefore, while prolonging gestation in preterm births by just 1 week has been estimated to have the potential to save $25 million in direct hospital costs [6], identifying risk factors for morbidity related to preterm birth and developing novel interventions to prevent them is also critical.

1.2 Morbidity and Mortality Associated with Prematurity

Preterm infants are one of the most vulnerable hospital populations [7]. In developed countries preterm birth accounts for 70% of deaths during the neonatal period [8]. All preterm infants, but particularly those born extremely premature, are susceptible to a number of potentially fatal or severely disabling conditions including bronchopulmonary dysplasia (BPD), necrotising enterocolitis (NEC), intraventricular haemorrhage (IVH), retinopathy of prematurity (ROP),

periventricular leukomalacia (PVL), sepsis and anaemia [9]. Importantly, all of these conditions are associated with long-term physical and neurodevelopmental consequences [9]. While the mechanism of each condition is not fully understood, and with all likely multifactorial, they all have an inflammatory basis [10-13]. Therefore, inflammation could be the linking mechanism of neonatal morbidity and mortality.

1.2.1 Bronchopulmonary Dysplasia

BPD or chronic lung disease is characterised by the need for supplemental oxygen at 36 corrected weeks as a result of disrupted alveolar growth [10,14]. It is the predominant neonatal morbidity that occurs as a consequence of prematurity [14]. BPD predisposes infants to significantly higher rates of cardiopulmonary, vision and hearing impairments, growth failure, neurodevelopmental delay and more importantly, neonatal mortality [14]. While studies have identified that preterm infants suffer less severe chronic lung disease with decreased risk of mortality than what was originally observed and described by Northway [15], the incidence in surviving infants born at less than or equal to 28 weeks' gestational age has been relatively stable at approximately 40% over the last few decades [16].

The exact mechanism that underpins BPD is not fully understood. However, there are a number of factors that contribute to the pathogenesis such as lower gestational age and exposure to mechanical ventilation. It is now acknowledged that inflammation has a central role, particularly exposure to prenatal and postnatal infection. The contribution of inflammation is supported by the association between BPD and increased plasma levels of Interleukin (IL)-1β, IL-6, IL-8, and IL-10 [10] and appears related to an inability to mount an effective anti-inflammatory response [10]. While aberrant cytokine production is associated with BPD, it has been proposed that some cytokines actually have a protective role, in particular macrophage migration inhibitory factor (MIF) [17]. In animal models, MIF has been shown to influence and accelerate immature lung development [17]. In infants who develop respiratory distress

syndrome (RDS) there is significantly less circulating MIF [17] [18]. However, MIF is an upstream regulator of the innate immune system and has been implicated in a number of inflammatory conditions in adults highlighting a complexity of roles in the balance between injurious and beneficial cytokine production.

1.2.2 Necrotising Enterocolitis

NEC is an inflammatory condition of the gastrointestinal system, predominantly seen in preterm or LBW infants [19] with a reported incidence of between 3-15%. Importantly, despite advances in neonatal care, little progress has been made in the treatment of NEC in recent decades, partly due to the unclear aetiology. Recognised risk factors for the development of NEC include prematurity, growth restriction and exposure to hypoxic ischemic events [20]. The highest mortality rate is in those infants requiring surgery for NEC with 1 in 3 infants dying within the first postoperative year [21]. While the exact pathogenesis of NEC is not fully understood, as for BPD it is considered to be multi-factorial. Recognised factors that play a role in its pathogenesis include immaturity of the gut, hypoxia-ischemia, microbial dysbiosis and a vulnerable gut barrier with impaired inflammatory defence mechanisms [20].

Fundamentally, NEC is the end result of a disruption to the integrity of gut barrier defences [20]. The gastrointestinal system (GI) is unique in its barrier defence mechanisms with the epithelial lining predominated by enterocytes, absorptive cells which act as the physical barrier between the lumen, GI tract and the lamina propria [11]. Enterocytes are integral in mucosal immunity and defence. The homeostatic state is achieved through a closely monitored cycle of proliferation, migration and apoptosis of enterocytes, which occurs every 3 to 5 days [11]. If there is disruption to this cycle, then bacterial translocation may occur, resulting in vulnerability to intestinal injury [11].

While the preterm infant's immune system is inherently immature, it has been proposed that by 23 weeks gestation the gut is already equipped with immune regulatory mechanisms [11].

However, in tissue samples from both animal models and preterm infants with NEC, Treg number is significantly reduced. In addition, there are increased levels of pro-inflammatory cytokines, in particular T-helper (Th) 17 family cytokines and IL-6 which is required for Th17 cell expansion and stabilisation. The critical role of the Th17 family of cytokines is supported by murine models deficient in functional T and B cells where intestinal injury and mucosal cytokine production is greater, secondary to increased production of Th17 cytokines and disruption of the enterocyte homeostatic cycle [11]. This was characterised by weakened enterocyte junctions, increased enterocyte apoptosis and decreased enterocyte proliferation [11].

1.2.3 Intraventricular Haemorrhage

While there is a recognised link between conditions such as NEC and BPD with inflammatory cytokines, the association between inflammation and IVH is not as clearly established. IVH is categorised by a grading system, with the most severe grades 3 and 4 characterised by blood within the ventricular spaces with prominent ventricular dilation and/or haemorrhage with parenchymal infarction [22] and associated with significant morbidity and mortality [23]. Recognised risk factors for IVH include preterm delivery without administration of antenatal glucocorticoids, lower gestational age, and sepsis [23]. Approximately 90% of IVH occurs within the first 72 hours of birth [24] with severe IVH associated with higher risk of mortality and significant long-term morbidity such as cerebral palsy, hearing and vision deficits and later learning disability [25]. Despite advances in neonatal care resulting in improved survival, there has been little impact on the prevalence of IVH [26].

Increased concentrations of IL-6 in cord blood and maternal peripheral blood have been associated with the development of IVH [12]. This is thought to be due to an interaction between inflammation and the microvasculature [12, 27]. Systemic inflammation not only independently induces damage but is also able to initiate a cascade of events within the central nervous system (CNS). In a pro-inflammatory state, the microvascular endothelial cells contained within

the CNS are capable of upregulating inflammatory cells, triggering neutrophil chemotaxis and attachment, ultimately damaging surrounding tissue [27]. The damaged endothelium is then able to promote the upregulation of adhesion molecules such as soluble intercellular adhesion molecule (sICAM) and vascular cell adhesion molecule (sVCAM) initiating a cascade of additional pro-inflammatory processes [28, 29]. Further, this upregulation results in vasoparalysis and damage to the germinal matrix and white matter injury [12, 27].

1.2.4 Retinopathy of Prematurity

A meta-analysis of 13 population-based studies over the past decade from countries with a neonatal mortality rate <5 per 1000 births reported 22% (95% CI 17–27%) of infants of <32 weeks' gestation develop ROP of any stage [30]. The most recent Australian and New Zealand Neonatal Network (ANZNN) report shows that severe ROP (stages 3 or 4) occurs in 5–6% of infants with a gestational age <31 weeks or birthweight <1250g. Treatment is administered to roughly half of these, with the most severe disease confined to infants of <27 weeks' gestation [31].

The pathogenesis of ROP is not primarily thought to be a consequence of inflammation but the result of excessive and prolonged oxygen therapy [32]. It is a unique preterm condition affecting retinal development and is the main cause of visual impairment and blindness [33]. Retinal development typically begins during the fourth month of gestation and therefore preterm infants are born with a highly underdeveloped visual system [34]. Numerous cell types play critical roles in retinal development including endothelial cells, astrocytes and microglia [32].

There are two important stages in retinal development which involve the signalling cytokine vascular endothelial growth factor (VEGF) [35]. During normal retinal development, astrocytes are the key drivers. These cells are highly sensitive to hypoxic conditions which results in them releasing VEGF [32]. The release of VEGF stimulates vasculogenesis and angiogenesis [32]. VEGF

initiates the proliferation of new blood vessels towards the VEGF source [34]. Therefore, new blood vessel formation occurs changing the predominantly hypoxic state to a hyperoxic one [34]. In the presence of hyperoxia, VEGF mRNA expression is ceased resulting in discordant vascularisation [34]. Supplemental oxygen inhibits the necessary hypoxic state leading to a reduction in VEGF production causing damage or destruction of the vasculature. This is considered to be phase one of ROP development. In phase 2, there is upregulation of VEGF production causing discordant vascular growth [34]. While not thought to be primarily a consequence of dysregulated inflammation, excessive VEGF release is shown to be substantially increased by high IL-6 [13].

In summary, the pathogenesis of these significant morbidities is clearly multi-factorial. However, all have been shown to be associated with inflammation. Importantly, for each of these, exposure to a red blood cell (RBC) transfusion during the neonatal period has been identified as an independent risk factor for their occurrence [36]. A common pathway linking susceptibility to these morbidities is likely to be increased pro-inflammatory cytokine production in response to packed RBC (PRBC) exposure [37, 38]. This raises the possibility that the pro-inflammatory response seen in the transfused recipient could be a common contributing pathway leading to the development of inflammatory morbidities. Clearly, with the knowledge gaps identified for each morbidity, there is a pressing need to increase our understanding of how a transfusion influences morbidity and mortality outcomes in preterm infants.

1.3 Anaemia of Prematurity

A decline in circulating RBCs is observed in all newborn infants after the first 24 h of age and continues to fall over the first weeks of postnatal life [39, 40]. This postnatal decrease in haemoglobin (Hb) is termed physiological anaemia of infancy [41]. However, unlike term infants, in the preterm infant this fall is exaggerated and more rapid with a nadir at 4–6 weeks postnatal age [42]. This is due to a smaller circulating blood volume [43], a shorter RBC lifespan compared to

that of an adult and an immature bone marrow response to anaemia resulting in insufficient RBC production [44]. Additionally, a premature infant is deprived of placental iron transport from the mother during the final trimester of pregnancy [41]. In addition, and directly related to the degree of prematurity, iatrogenic blood loss due to repeated blood sampling as part of routine neonatal care is a significant contributor to the development of anaemia in the preterm infant and further exacerbates the increased risk of physiological anaemia [44].

Term infants are generally asymptomatic from this physiological anaemia however in preterm infants the fall in Hb is commonly accompanied by physiological symptoms including feeding difficulties and poor weight gain, apnoea, tachycardia, hypotension and lethargy [39, 42]. This is collectively termed anaemia of prematurity (AOP) and represents a pathologic extreme of physiologic anaemia in a high-risk population of newborns. Further, it is the commonest reason for a transfusion in the neonatal period [42].

While there are a number of strategies employed to minimise the decrease in Hb, such as delayed cord clamping [44], preterm infants remain susceptible to AOP. As a result, PRBC transfusion is used to preserve oxygen carrying capacity to tissues, replace blood volume, prevent apnoeic episodes and foster weight gain and growth [39, 45]. Ninety percent of all LBW premature infants will develop AOP and will receive at least one PRBC transfusion [41], however a number will require multiple transfusions (3-5) during their primary hospital admission [46]. The decision to transfuse an infant with AOP is generally centred upon the use of a transfusion algorithm based on either Hb or haematocrit, modified by chronologic age and need for respiratory support [41, 47, 48]. To date, the question of what constitutes the safest transfusion threshold in the preterm newborn remains unanswered.

This thesis will explore potential pathways through which PRBC transfusion could result in adverse neonatal outcome and propose a novel approach to improve transfusion safety in this high-risk preterm population.

1.4 Transfusion Associated Mortality and Morbidity

1.4.1 Mortality and Transfusion Exposure

Considerable evidence suggests that transfusion is associated with an increased risk of death in critically ill adult patients. Studies have reported associations between low volume PRBC transfusion [49], increased PRBC transfusion exposure [50] increasing age of transfused RBCs [51] and increased short and long-term mortality risk [52]. More limited evidence supports a similar association in the paediatric population [53]. Preterm infants are one of the most heavily transfused patient groups [54] and represent one of the most vulnerable hospital populations with preterm birth accounting for 70% of deaths during the neonatal period [8]. Despite this, neonatal data linking RBC transfusion exposure and increased mortality is scarcer.

Dos Santos, in a large retrospective observational study of preterm infants <1500 grams (n= 1077), reported an approximately 50% increase in intra-hospital mortality in infants who received any leukodepleted PRBC transfusion in the first 28 days of life after adjusting for confounding variables. Further, mortality after day 28 of postnatal life was almost 2 times greater in infants who received 2 or more transfusions [55]. Similarly, in a recent cohort study in ELBW infants, the number of PRBC transfusions in the first 7 days of life correlated with mortality rate before 1 month of age [56]. However, other data supporting an association between exposure to leukodepleted PRBC transfusion in infants <30 weeks' gestation (n= 490), after 21 days postnatal age and significant neonatal respiratory morbidity did not find an association with mortality [57].

The proposed mechanisms underlying the association between RBCs and post-transfusion mortality remain poorly understood. While transfusion-associated sepsis, transfusion-related

acute lung injury, and haemolytic reactions are the leading causes of allogeneic blood transfusion-related deaths in adults [58], they are seldom recognised in infants. Some authors have suggested that PRBC transfusions are associated with pro-inflammatory and immunosuppressive responses, together termed transfusion-related immunomodulation (TRIM), which contribute to the development of multi-system organ failure [59]. TRIM represents a biologically plausible, pathophysiologic process linking PRBC transfusion exposure and subsequent morbidity and mortality. As described previously, extremely premature infants are susceptible to numerous potentially fatal or severely disabling conditions, including NEC, BPD, ROP and IVH, all of which have been shown to be related to inflammation. While a number of observational studies have raised the possibility of an association between PRBC transfusion and adverse neonatal outcome, the extent to which transfusion exposure contributes to their pathophysiology is unresolved and controversial [60]. The significance and mechanistic basis of transfusion-related neonatal morbidity is inadequately addressed in the medical literature and represents a significant knowledge gap.

1.4.2 Necrotising Enterocolitis and Transfusion Exposure

NEC typically develops in a vulnerable preterm host with a hypoxic, ischemic, or infectious bowel insult. In the 1980's, the association between PRBC transfusion and NEC was thought to occur as a result of T-crypt-antigen activation, leading to the use of washed or anti-T blood products [61]. Recently the term TANEC has been used to describe transfusion-associated necrotising enterocolitis. TANEC has been reported to be associated with approximately 30% of NEC cases, with these infants at greatest risk of developing surgical NEC [62]. While a number of mechanisms have been proposed to result in TANEC, it is generally thought to be secondary to an immune response to biological mediators contained within stored blood [62]. Indeed, PRBC transfusion exposure results in increases in pro-inflammatory cytokines associated with established NEC including tumour necrosis factor (TNF), IL-1β, IL-6, and IL-8 [63].

Other investigators have focused on the temporal relationship between enteral feeding at the time of PRBC transfusion and the development of NEC, demonstrating that the anticipated postprandial blood flow increase through the superior mesenteric artery is ablated in infants receiving PRBC transfusions. The resultant postprandial period of relative intestinal hypo-perfusion may then contribute to the development NEC [64]. Finally, it has been suggested that anaemia itself, rather than a transfusion, increases the risk of NEC [64]. This hypothesis is supported by evidence from a prospective observational study (n= 598) of very low birth weight preterm infants (<1500 grams) in whom PRBC transfusion in a given week was not significantly related to the rate of NEC. However, the rate of NEC was significantly increased in those infants with severe anaemia compared to those without anaemia [65].

While a previous meta-analysis of eleven case-control and one cohort study suggested an association between PRBC transfusion and NEC [66], the most recent meta-analysis of available observational data found no such effect [67]. Further, a recent systematic review of randomised trials on blood transfusion thresholds in ELBW infants failed to support an association between transfusion and NEC [68]. Rather than supporting a lack of association between PRBC transfusion and NEC, this likely reflects that most available studies are affected by unadjusted differences in baseline patient characteristics and indication for transfusion, the lack of a specific definition of TANEC and, critically, significant inconsistency in the direction of results [68].

1.4.3 Bronchopulmonary Dysplasia and Transfusion Exposure

In adults, acute respiratory decompensation is a well-recognised complication of PRBC transfusion [69]. This may be either transfusion-associated lung injury (TRALI), commonly occurring 6 hours following exposure to RBCs [70], transfusion-associated circulatory overload (TACO), defined as acute respiratory distress, tachycardia, elevated blood pressure, acute or worsening pulmonary oedema and evidence of positive fluid balance, or transfusion-associated dyspnoea, a clinical condition not meeting the proposed diagnostic criteria for TRALI or TACO

[71]. TRALI is one of the leading causes of severe post-transfusion morbidity and acute mortality [72]. However, TRALI is thought to be underreported and under-recognised in all patients, particularly in preterm infants [73]. The incidence of TRALI has been reported to vary from 0.08% to 15% of adult patients receiving a transfusion [74] but the incidence in neonates is unknown. A two-event hypothesis has been postulated as central to the pathogenesis of TRALI [75]. The first event is the patient's underlying clinical condition, causing inflammation with priming of the pulmonary neutrophils. The second event is the transfusion product itself, with bioactive molecules or antibodies which have accumulated during RBC storage, activating primed neutrophils in the recipient [75]. Although TRALI has previously been described in the paediatric population [73] there is a paucity of data in neonates.

BPD is among the most common and serious sequelae of preterm birth. This chronic condition is characterised by the need for supplemental oxygen at 36 corrected weeks and is the result of disrupted alveolar growth. BPD is inherently multifactorial; therefore, there is no consensus on the precise pathogenesis. Interestingly, the incidence of BPD is significantly higher in infants transfused during the neonatal period [76] and increases with the total number of transfusions [76]. There is growing recognition of the significant contribution of inflammation and oxidative stress to the pathogenesis of BPD, processes similar to that seen with TRALI [77]. While rates of post-transfusion lung injury (defined as a sustained increase in mean airway pressure of >2cm or FiO_2 >0.15 occurring within 6 hours post transfusion exposure and persisting for up to 18 hours post exposure) are reported to be as high as 10% in preterm neonates, failure to take into account very high rates of respiratory instability in this population limits interpretation of this data [78]. In a retrospective study of 108 infants transfused a total of 373 times the rate of post-transfusion respiratory deterioration was 8.3% [78]. However, meta-analysis of randomised trials comparing restrictive versus liberal transfusion thresholds fails to demonstrate an increased incidence of chronic lung disease with greater transfusion exposure [60]. It is important

to acknowledge that both observational and randomised studies are limited by the lack of an accepted definition of TRALI in the preterm infant in addition to the difficulty in differentiating the contribution of underlying lung pathology versus a transfusion-related effect. As a result, prospective studies with significantly larger sample sizes are required to definitely prove or disprove any association [79].

1.4.4 Retinopathy of Prematurity and Transfusion Exposure

Red cell exposure and the development of ROP remains controversial. A number of observational studies support an association with RBC exposure and ROP [80-82]. Most report a significant association between both gestational age and transfusion frequency with the development of ROP [80, 82]. Others report a significant association between transfusion exposure and ROP following adjustment for gestational age [81]. Hesse and colleagues adjusted for gestational age and duration of mechanical ventilation in a population of ELBW infants and found the relative risk for ROP development was 6.4 (95% CI 1.2 to 33.4) in those who received 16-45 ml/kg of RBCs, doubling for those receiving >45 ml/kg over their admission [83], an effect proposed to be associated with products of iron metabolism [82]. Conversely, the sole randomised study investigating the relationship between transfusion exposure and the incidence of ROP failed to demonstrate an increase in the risk for ROP in those infants allocated to the liberal transfusion threshold arm [84]. However, this study is confounded by high attrition rate overall, variability in haematocrit in the liberal transfusion arm and very small sample size for a study with a clinical outcome. Unsurprisingly, as for BPD, meta-analysis of randomised trials comparing restrictive versus liberal transfusion thresholds fails to demonstrate increased incidence of ROP with greater transfusion exposure [60].

Unlike NEC and BPD, the underlying pathogenesis of ROP is not primarily a consequence of inflammation. Exposure to biologically active substances from the transfusion pack may participate in redox reactions and oxidative damage but whether these transient effects are

significant enough to contribute to the pathogenesis of ROP remains unknown [85]. Other potential mechanisms linking transfusions with ROP development include lowering the ratio of fetal haemoglobin (HbF) to adult Hb with transfusion exposure, shifting the oxygen dissociation curve to the right and increasing oxygen availability to the developing retina [86]. Finally, anaemia itself may result in retinal hypoxia and subsequently retinal neovascularisation [84].

1.4.5 Intraventricular Haemorrhage and Transfusion Exposure

An association with PRBC transfusion exposure was first reported in 1998 [87] with each subsequent PRBC transfusion in the first week of life in ELBW infants doubling the relative risk of severe IVH [23]. Further, in a retrospective study of 417 infants already diagnosed with a grade 1 IVH, subsequent transfusion exposure, along with lower gestational age, was the most significant predictive factor in the 46 infants who subsequently developed grade III or IV haemorrhage [88]. Similarly, in a prospective cohort of 4283 preterm infants, those who received a transfusion had a 64% greater chance of diagnosis with grade III/IV haemorrhage [89]. There is also evidence supporting an association between PRBC transfusion and IVH from a transfusion compliance study. Following the implementation of a standardised transfusion approach which was associated with a 23% reduction in the number of very low birth weight infants transfused, the incidence of severe IVH decreased from 17% to 8% [22].

However, interpretation of results from retrospective studies is limited by their failure to adequately deal with confounding variables and the inherently greater risk of bias. For instance, the association between transfusion and IVH is likely related to the clinical reason for which the transfusion was required versus the transfusion itself. If the relationship between PRBC transfusion exposure and subsequent IVH is truly causal then any intervention that reduces or prevents the need for early PRBC transfusion should be associated with a reduction in IVH occurrence [90]. One such approach is delayed cord clamping, resulting in both reduced need for transfusion and lower incidence of IVH [91]. However, the results of the largest study investigating

this intervention on neonatal outcome failed to show a significant reduction in IVH rates in the delayed cord clamping group [92]. As such, the latest meta-analysis of available studies (including the APTS study [92]) reports no difference in incidence of IVH despite a 10% reduction in transfusion exposure in infants randomised to delayed cord clamping [93].

1.4.5 Summary

Does PRBC transfusion contribute to development of neonatal morbidity? The majority of data supporting an association between PRBC transfusion and adverse outcome comes from small retrospective and observational studies. Consequently, it is important to acknowledge limitations of much of the primary evidence. This includes the failure to recognise confounding variables and limitations of the studies due to their susceptibility to bias. Therefore, while retrospective investigations may be hypothesis generating and report associations, they rarely conclusively prove a causal relationship.

The most recent systematic review of PRBC transfusion exposure and adverse outcomes in the preterm neonatal population investigated clinical adverse effects and associations attributable to RBC exposure in 61 randomised and non-randomised trials [60]. The authors concluded that there was no evidence that mortality risk was greater between liberal or restrictive transfusion thresholds. However, they cautioned that many studies were likely confounded by indication bias, with the more critically ill infants more likely to receive PRBC transfusion [60]. While approximately one-third of identified studies were randomised, the sample sizes in many were considered inadequate to address harm [60]. Importantly, they conclude there is a "pressing need for larger studies with clear definitions of adverse events to be conducted prospectively, so that uncertainty about the safety of transfusion can be addressed in a population of recipients characterised by prematurity and relative immunologic immaturity" [60].

1.5 Transfusion Related Immunomodulation

The proposed mechanisms underlying the association between RBC and the spectrum of post-transfusion multi-organ morbidity and mortality remain poorly understood. Numerous pre-clinical studies demonstrate that RBC products can directly modulate immune cell function [59]. This may represent a common pathway linking RBC exposure to adverse outcomes, a process termed TRIM [94]. TRIM describes both adverse pro-inflammatory and immunosuppressive responses. In the preterm neonate it may be the central process underlying TRALI; associated with an increase in BPD and TRAGI; linked to an increase in the occurrence of NEC [95].

TRIM was first described in the late 1970's by Opelz and Terasaki. They discovered that as the number of transfusion exposures increased kidney graft survival improved suggesting that transfusion produced an immunosuppressive response [96]. However, subsequent in vitro and clinical trials have demonstrated a transfusion has both pro- and anti-inflammatory potential. TRIM has been proposed to be a "two-insult" process. While two independent insults alone are harmless, sequential insults can cause pathophysiologic inflammation [97]. Initial sensitisation to inflammatory processes results in priming of host neutrophils (first insult). Subsequent exposure to biological response mediators passively transfused along with the RBCs results in a pro-inflammatory immune response (second insult) [98]. In vitro data supports the ability of the supernatant from stored RBCs to "prime" unstimulated allogeneic neutrophils inducing the release of IL-8 and altering the cells chemotactic properties [99]. Further, TRIM appears to be characterised by activation of vascular endothelial cells and platelets. These cells are highly sensitive to inflammatory signals and may release toxic bioactive mediators following activation by donor blood [98]. This can result in recruitment of suppressor T cells (that are important in regulating pro-inflammatory immune responses), apoptosis of immune cells, and the accumulation of immune molecules, ultimately inhibiting neutrophil function [100, 101].

While these pathological processes are yet to be fully characterised, the biological response mediators implicated in the second "hit" may include donor antibodies, bioactive lipids, free haemoglobin, red cell membrane fragments and cytokines that accumulate during blood product storage [95]. Through normal storage processes (PRBCs stored for up to 42 days and kept at 6 degrees) the RBCs undergo structural and biochemical changes resulting in 'shedding' of an outer membrane, a process exacerbated as storage time increases [102]. As homeostasis of the red cell is lost through normal erythrocyte aging the phospholipid membrane is disrupted, adenosine triphosphate (ATP) is depleted, and oxidation and haemolysis occurs. This results in the shedding and accumulation of microparticles and other inflammatory cells in the supernatant, a process commonly referred to as "storage lesion" [102]. Whilst the inflammatory cells within the supernatant induce an inflammatory response themselves, microparticles indirectly stimulate immune responses through the recipient's antigen presenting cells inducing a T-cell immune response [103]. When a patient is transfused macrophages attempt to clear the RBC microparticles inducing an inflammatory response [102, 103]. In vitro studies have shown that when stimulated, mononuclear cells significantly increase the production of more than 20 cytokines and chemokines including IL-1α, IL-1β, IL-17 and TNF [103]. All of these have been identified as important cytokines in the underlying pathophysiology of a number of neonatal morbidities.

Non-transferrin bound iron (NTBI) has also been identified as a bioactive substance which may contribute to TRIM [85]. NTBI is detected in PRBC transfusion packs as early as day three of storage [104, 105] and is a potential contributor to the development of transfusion-associated morbidities [85]. In the transfusion recipient, the accumulation of NTBI is prevented by caeruloplasmin and transferrin. However, this enzyme and protein are significantly reduced in prematurity [85]. In a mature circulating system, when free iron is detected caeruloplasmin converts pro-oxidant ferrous into a ferric state allowing transferrin to bind the ferric iron, in turn, reducing any free circulating iron. If caeruloplasmin is depleted, transferrin is maximally

saturated resulting in NTBI accumulation [105]. This may result in the production of reactive radicals, resulting in oxidative damage and cytokine production [85].

Newborn infants represent a unique patient population with the neonatal immune system, particularly one preterm, very different from adults [106]. As a result, while a two-insult process may be common across all age groups, what induces TRIM in the preterm infant may be very different from the pathophysiologic process in adults. Further, the clinical endpoint may also differ. For instance, while TRALI may represent the commonest transfusion related morbidity in more mature patients, TRAGI presenting as NEC may be more common in the preterm infant. For instance, the mucosa of the naïve neonatal gut is particularly rich in neutrophils and on first exposure to gut flora and /or nutrient antigens is prone to an exaggerated immune response with a conversion from a TH2 to TH1 phenotype involving primed neutrophils [107]. Nevertheless, it is clear that the immune response to transfusion exposure in the preterm infant, and how this relates to later morbidity, remains a significant knowledge gap.

While TRIM has not been definitively proven in preterm infants, they are potentially at greater risk of immunomodulation due to their immature immune system. The in utero environment is generally proposed to be sterile with immune protection of the fetus predominantly of maternal origin [106]. Initially, the neonate relies solely on their innate immune system to defend against potential pathogens [106]. This is principally the ability of circulating neutrophils to phagocytose and respond to pathogens [106]. Neonatal neutrophils are markedly different to their adult counterparts; their numbers are less than 10% of circulating adult neutrophils and they have reduced adhesion and phagocytic ability [106]. Further, these differences are exaggerated with increasing prematurity. As a result, the preterm infant is uniquely vulnerable to sepsis [106]. This vulnerability is compounded by the fact that the neonatal immune response

is thought to be biased towards a Th2 cell response [108] with a predominantly anti-inflammatory response observed during the neonatal period [109].

In healthy term infants the innate immune response does not tend to result in a Th17 response [110]. This cytokine family is important in mediating chronic inflammation. An inflammatory environment influences the differentiation of naïve Th cells into the Th17 lineage [111]. Th17 cell numbers are initially very low in the neonatal circulation. However, IL-6, IL-21, IL-23 and TGFβ predominate in the early neonatal period and promote maturation and stabilisation of Th-17 cell expansion [112]. Through the production of IL-17[113], Th17 cells subsequently aid in the recruitment of phagocytic cells, primarily neutrophils and predominantly at mucosal surfaces [112, 113], enabling future adaptive immune responses [109].

Importantly, excessive production of IL-17 has been implicated in certain inflammatory disease conditions including inflammatory bowel and lung disease [114]. In adult patients with chronic lung disease those with the most severe disease had the highest expression of Th17 mRNA and protein [112]. High concentrations of TGF, IL-6, IL-23 and/or IL-21 result in increased ROR-c expression, a transcription factor that induces the Th17 phenotype [111]. Sustained IL-23 production stabilises the Th17 cell enabling continued production of Th17 cytokines IL-17A, IL-17F, IL-21 and IL-22 [111]. It is thought that the Th17 lineage is regulated differentially according to maturity, such that preterm infants may not have the ability to balance anti- and pro-inflammatory responses in the neonatal period resulting in over-production of Th17 cytokines [113].

Perinatal and early life events are known to contribute to the development of immune disorders later in life. This is likely secondary to programming of the neonatal immune system, influencing future immune function and response [115]. Perinatal exposures implicated in the

programming of the neonatal immune system include maternal infection, tobacco exposure, growth restriction and chorioamnionitis [116]. The balance in T helper cell response is essential for maintaining immune homeostasis and when disrupted results in disturbances to the normal cellular and humoral immune regulation [116]. Chorioamnionitis, for example, has been linked to postnatal chronic inflammatory conditions, such as BPD, NEC and ROP.

Animal data supports the hypothesis that the hyper-inflammatory environment caused by exposure to intra-uterine inflammation results in impaired innate and adaptive immune interactions, disrupting the homeostatic environment [115]. In a murine model of intra-uterine inflammation employing exposure to low-dose lipopolysaccharide, the result is a profound pro-inflammatory response extending beyond the original infected tissue [115]. This inflammatory response reflects a change in T-cell response from a predominantly anti-inflammatory Th1/2 response to one far more damaging. This is thought to be due to plasticity of the neonatal lymphocyte favouring a Th17 response, resulting in a change from a T-regulatory response to a pro-inflammatory response. Subsequently, the alteration to this adaptive pro-inflammatory phenotype alters the trajectory of future immune responses.

2 Modifications to Transfusion Practice

Our growing understanding of TRIM and the relationship between transfusion exposure and significant morbidity and mortality has led to a number of different approaches designed to minimise the risk of adverse outcomes following PRBC transfusion being trialled and subsequently adopted into clinical practice. Modifications to transfusion practice and to RBC processing have both been areas of active research. Interventions have included the use of restrictive versus liberal transfusion triggers [48], the transfusion of fresh versus older RBCs [117], and pre-storage leukodepletion [118]. However, these studies have had varying degrees of success in reducing transfusion-related adverse outcomes.

2.1 Minimising Transfusion Exposure

Transfusion therapy represents a unique balance between predisposing patients to potential transfusion related adverse outcomes whilst managing the clinical consequences related to anaemia. As a result, transfusion protocols are inherently ill-defined. Many are based on a predetermined haemoglobin or haematocrit threshold, while others rely on patient signs and symptoms [119]. For many years there has been a decrease in the number of transfusions patients are exposed to as a result of an increased understanding of transfusion-associated morbidities. Consequently, decreases in haemoglobin are increasingly tolerated. Despite this also being the case for newborns, preterm infants remain a particularly heavily transfused population [120].

There is little consensus on an acceptable or tolerable fall in haemoglobin in the newborn infant and therefore inconsistencies exist between hospitals and clinicians [120]. Much concern remains regarding the potential adverse consequences of chronic anaemia, particularly its effect on growth in extremely preterm infants [121] with blood transfusions associated with improved weight gain. As a result, there remains a fine balance between the risk of harm from anaemia itself and the risk of transfusion-associated morbidities.

Adherence to transfusion guidelines decreases exposure to donor blood and therefore possible adverse outcomes. Preterm infants cared for in clinical environments which do not have transfusion guidelines are two times more likely to receive a blood transfusion than those that are [122]. Therefore, much attention has been directed towards understanding and reaching a consensus on acceptable limits for falling haemoglobins in the preterm population and the identification of a transfusion "threshold". A restrictive threshold requires that a patient's haemoglobin or haematocrit falls below a specific value before a transfusion is "triggered". In adult patients a commonly used restrictive threshold is 7 to 8 g/dL. The use of a specific threshold results in both fewer transfusions and a lower total volume of blood exposure.

Conversely, a liberal threshold allows for smaller falls in Hb concentration (10 to 11 g/dL). Current Australian medical guidelines state that the evidence is unclear whether one transfusion threshold provides more benefit than another [123].

While no benefit of a restrictive transfusion threshold over a more liberal threshold has been demonstrated in adults [124], whether adoption of a restrictive approach is associated with an increased risk of harm remains unresolved[125-127]. Studies in cardiac and oncology patients have reported no benefit in a restrictive haemoglobin transfusion threshold [122, 126] with no difference in length of hospital stay, or incidence of organ dysfunction or sepsis [124]. In fact, two studies show there were significantly more deaths in the restrictive group at 90 days post-surgery [124, 128]. The authors of both these studies concluded that for these patient populations the use of a restrictive threshold may place patients at greater risk of harm. Therefore, the question as to which is the best approach remains unanswered [124, 128]. Conversely, in burns patients, adoption of restrictive transfusion thresholds resulted in no difference in in-hospital or 90-day mortality[126]. Clearly the conflicting results described by these studies could be a result of the widely differing patient groups. Further, both the cardiac and oncology studies had a number of methodological limitations. In both the restrictive and liberal arms were only marginally different in haematocrit concentrations. In particular, the study conducted by Murphy et al applied a very small 1 g/dL difference between the liberal and restrictive arms. Therefore, both studies may have identified the increased risk of death associated with a transfusion rather than falling haemoglobin. In addition, the Murphy study does not state what type of blood is used [124]. This is of particular importance as a transfusion with standard blood has previously been shown to significantly increase the risk of morbidity and mortality, where the rates are significantly decreased with the use of leukodepleted blood [118, 129].

In paediatric intensive care patients, a restrictive versus liberal transfusion threshold has been reported to have no effect on the development of multiple organ dysfunction syndrome (MODS; including respiratory, cardiovascular, hematologic, hepatic, gastrointestinal, neurological and renal dysfunction) [130]. While a trend towards increased sepsis susceptibility was observed in those randomised to the restrictive arm of the study, this finding is inconsistent with other studies which report that as transfusion exposure increases, rates of infection and mortality also increase [118]. Karam et al. conducted a post hoc analysis on this same population specifically focussing on patients who had septic shock [131]. This is particularly pertinent due to the two-hit hypothesis of TRIM where the first hit involves sensitisation by an underlying inflammatory condition. While no difference in any of the morbidity outcomes between the restrictive and liberal arms was found, the study was not blinded. This can lead to bias with clinicians more likely to break protocol and transfuse or withhold transfusion based on their interpretation of the patient's stability.

Two clinical trials, comparing a liberal versus restrictive transfusion schedule based on Hb or haematocrit [47, 48] are the foundation of current neonatal transfusion practice. Bell and colleagues randomised 100 newborns weighing 500-1300 g at birth, to either a restrictive or liberal transfusion threshold based upon haematocrit [47]. This trial reported a difference in the primary outcome of number of transfusions with fewer in the restrictive group. However, secondary outcomes of in-hospital survival, ROP, BPD, time in supplemental oxygen or growth were not different. While a non-significant difference in the incidence of brain injury diagnosed on cranial ultrasound was reported, with rates higher in the restrictive arm, only half of the study cohort were examined.

The Preterm Infants in Need of Transfusion (PINT) study also randomised preterm neonates (<31 weeks and less than 1000 g) to a restrictive versus liberal transfusion threshold [48]. In a

sample size of 451 newborns no differences were reported in a composite measure of mortality and/or significant neonatal morbidity (defined as BPD, vision threatening ROP or brain injury on cranial ultrasound) between the restrictive and liberal arms. As a result, the investigators concluded that lowering the haemoglobin transfusion threshold was safe for low-birth-weight preterm infants [48].

Both studies also undertook longer term follow-up. The PINT study assessed the potential neurocognitive effects of transfusion thresholds by employing a combined measure of death and severe adverse neurodevelopmental outcomes at 18 months [132]. The authors found that there was a greater number of patients with a cognitive impairment in those allocated to the restrictive threshold [121]. However, studies have shown that cognitive development scales used in infancy are poor predictors of later cognitive development [133].

Nopoulos and colleagues compared brain structure and function in children enrolled in the original Bell study at 13 years of age comparing both the restrictive and liberal groups to a control group [134]. They found that despite the short-term outcomes favouring a more liberal transfusion approach, long-term outcomes suggested the restrictive threshold provided a protective element to neurodevelopment [134]. Patients in the liberal group had significantly greater loss of intracranial volume compared to their control term counterparts, while patients in the restrictive threshold did not [134]. However, the follow-up rate was less than 40% and the study was under powered to obtain this outcome. Interestingly, female participants had the greatest volume decrease, despite being born at a later gestation, a known protective factor for neurocognitive impairment. While infants in the restrictive group had a higher incidence of IVH and PVL, at 13 years they seemed to be spared from volume loss. Erythropoietin (EPO) was thought to contribute to the potential neurocognitive benefits demonstrated by the restrictive group, with EPO previously shown to have neuroprotective capabilities [135]. As part of the original study, plasma EPO levels were measured with patients randomised to the restrictive

group having significantly higher concentrations of EPO compared to those in the liberal group. It is important to note that this long term follow up only assessed brain structure and did not assess cognitive ability. While previous studies have shown that changes in white and grey matter strongly predict neurodevelopmental outcomes, by merely assessing a single aspect through cortex volume without cognitive and behavioural assessments it is difficult to predict what the true neurodevelopmental outcomes are.

The subsequent Cochrane meta-analysis of all available neonatal transfusion threshold studies concluded that from the limited long-term data available, there was no clear benefit to the use of liberal versus restrictive Hb thresholds in very low birth weight neonates [136]. However, given the growing evidence supporting an association between PRBC transfusion exposure and adverse outcome in the neonatal period, it could be argued that a restrictive transfusion practice is preferable as more extremely preterm infants avoided transfusion without a worse outcome [66]. An alternate interpretation may be that a liberal transfusion practice is better due to the potential for a longer-term neurodevelopmental advantage [132].

2.2 Limiting Red Blood Cell Storage Time

Following donation, national guidelines require all RBC packs to be stored for no longer than 42 days. However, for more vulnerable populations such as neonates, current guidelines suggest, where possible, they should only be exposed to RBCs stored for a maximum of 2 weeks [123]. These guidelines are employed due to both morphological changes in the RBCs and accumulation of bioactive substances in the RBC packs as they age. After 2 weeks of storage the RBC is no longer a biconcave disk [137]. Following 3 weeks of storage it is irreversibly damaged, resembling a deformed sphero-echinocyte [137]. These structural changes alone have been identified as an independent risk factor for morbidity risk in trauma patients [97, 137]. Further, these changes are associated with red cell lysis and loss of membrane integrity, a decrease in pH, ATP and 2,3-Diphosphoglycerate (2,3-DPG), and an increase in oxidative damage and

production of reactive oxygen species (ROS) [98]. There is concurrent accumulation of bioactive substances within the pack supernatant with an increase in lactate, extracellular potassium, RBC microparticles and cell debris [98]. Collectively called "storage lesion", these changes are thought to contribute to TRIM and the increased risk of adverse clinical outcomes seen following a transfusion. Current storage practices are aimed at minimising these changes through optimising storage procedures and therefore increasing the time allowed for storage. In spite of these guidelines, there is long-standing debate on the optimal storage time and whether increased storage mitigates the benefits of a blood transfusion [138].

2.2.1. RBC Storage Lesion

As the red cell breaks down cell debris and microparticles accumulate. These are thought to exhibit immunomodulatory potential. Microparticles are able to attach to or near the endothelium allowing free nitric oxide to be scavenged [102]. This can result in platelet aggregation, endothelial activation and the production of ROS [102]. Microparticles have been identified as important contributors in a number of biological processes such as inflammation, immune function and apoptosis [139]. Microparticle generation results from a change in the asymmetry of the lipid bilayer of the cellular membrane. Physiological microparticle generation takes place concomitant to apoptosis of different cells and the presence of microparticles in the blood of healthy individuals is seemingly constant [140].

Microparticle generation may also arise due to physiological/pathological events resulting from cell activation by agonists[141]. During red cell storage several factors trigger microparticle formation. These include shear stress (due to the close contact between RBCs in the storage bag), anticoagulant-dependent effects, oxidative stress, calcium index and pro-apoptotic stimulation [140]. It is also known that the storage length is critical to microparticle formation. In addition, donor age and sex have been described to influence microparticle release upon storage, with blood from female or older donors more prone to microparticle formation [142].

Erythrocyte derived microparticles act on the innate immune system as paracrine messengers and as pro-inflammatory mediators inducing or propagating inflammatory signals [143]. It has recently been postulated that microparticles are likely to be mediators for TRALI through the binding and activation of neutrophils [144]. Further, complement and IgG are enriched in microparticles from "old" RBCs due to the storage lesion and can activate neutrophils via Fc receptors. In addition, microparticles exhibit potent pro-coagulant activity through the expression of anionic phospholipid phosphatidylserine (PS), which is critical for assembly of coagulation factors into active complexes for thrombin generation [145].

A pro-inflammatory response in the transfusion recipient, characterised by increased production of pro-inflammatory cytokines, is likely to be a significant component of the pathological processes resulting in transfusion-related morbidity and mortality. It would seem logical to expect that along with time-dependent accumulation of microparticles there might also be a progressive increase in cytokine concentrations as the blood pack ages [97, 98, 146, 147]. Bal et al found that whilst the majority of cytokines tested for were non-detectable, IL-17A progressively increased [147]. Interestingly, as IL-17A concentrations increased, Th1 expression was suppressed. This observation may partially explain the apparently contradictory findings in some in-vitro studies where pro-inflammatory cytokine concentrations and the pro-inflammatory response following exposure to supernatant from older packs was lower [148].

However, when untreated whole blood is exposed to supernatant from ‘fresh’ RBC packs (1, 6 or 15 days), IL-6 concentrations increase [97]. In a murine model Hod et al. found that fresher murine blood was still able to result in a significant inflammatory event. This pro-inflammatory response characterised by elevated IL-6, TNF, monocyte chemoattractant protein (MCP-1) and macrophage inhibitory protein-1β (MIP-1β) was even greater in those transfused with RBCs stored for longer than 14 days. Similarly, Baumgartner et al found that the ability of RBC

supernatant to accentuate LPS induced pro-inflammatory cytokine (IL-1, IL-6 and TNF) production increased with the age of the transfusion pack. Although in vitro studies have provided evidence for how length of storage affects inflammatory pathways, if and how this is translated clinically is far less definitive [51, 149].

2.2.2 Storage Lesion and Transfusion Associated Morbidities

Many studies have suggested a link between PRBC storage time and morbidity and mortality, although the evidence does not universally support such an association. Purdy et al, in 1997, reported the first evidence of an association between increased mortality rate and age of PRBCs transfused. In a cohort of adult intensive care patients diagnosed with sepsis the median age of PRBC units transfused to survivors was 17 days (range 5-35) vs 25 days (range 9-36) for those that died [150]. Offner et al found an association between the incidence of major infections in trauma patients (such as pneumonia, meningitis, lung, abdominal or pelvic abscesses) and the duration of red cell storage prior to transfusion [149]. This was thought to result from primed neutrophils previously found to occur following 2 weeks of storage [151]. When controlling for patient age, sex and mechanism of injury, those that received PRBCs stored for 14 days or 21 days had an independent increased risk for development of infection [149]. Similar findings were reported in a large single site study conducted by Koch et al which investigated the effects of transfusion storage duration and cardiac surgery outcomes [51]. Patients in this study were at an increased risk of developing a serious adverse event such as renal failure, multiple organ failure and sepsis, as well as a reduced survival rate [51]. Additionally, there was a 30% increased relative risk of post-operative death for those who received blood older than 14 days. It is important to note, however, that the length of storage time between the two groups did not differ substantially. Therefore, comparing a true fresh and older pack (>20 days of storage) remains an unanswered question.

An important consideration in the interpretation of this data is the potential confounding effects of repeat exposure, a known risk factor for transfusion associated morbidities and mortality. Of the 61 patients in the study conducted by Offner et al there were a total of 732 transfusions within the first 12 hours of the sustained injury [149]. This transfusion rate equates to an average of 12 units per patient which is extremely high. While transfusion exposure was lower for patients in the study by Koch et al, on average 3 transfusions per patient, additional analysis identified a dose response effect for the composite morbidity outcome [51]. Further, while both where large clinical studies, it is difficult to truly attribute increased morbidity and mortality risk solely to storage time as any identified associations may be an end product of volume exposure [152].

Importantly, some adult studies have failed to demonstrate any difference in outcomes [153]. For example, only a 0.3% difference in mortality rate and no difference in length of hospital stay was seen in a study of over 3000 patient outcomes when analysed by receipt of fresh blood compared to packs stored for a longer duration [153]. In fact, those exposed to packs with the shortest storage duration had a higher mortality rate. While not designed to investigate the underlying mechanism/s, the authors proposed that this could be related to significantly higher cytokine concentrations in 'fresher' packs described by earlier in-vitro studies [153].

Similar to studies conducted in adults, paediatric clinical studies report conflicting results. In older paediatric populations, increased storage time affects RBC cytokine concentration and clinical outcomes [97, 154]. Gauvin et al. found that the incidence of multi-organ dysfunction syndrome (MODS) was higher in patients who were transfused with older PRBCs (21 days) compared to those who received PRBCs stored for <14 days [154]. Similarly, Karam et al. found that patients who were exposed to older blood were more likely to develop MODS sooner than patients who were not transfused or transfused with 'fresh' PRBCs [97]. Conversely, when

preterm infants are exposed to either fresh or older blood, no differences are seen between morbidity or mortality outcomes [155]. It is important to acknowledge the generalisability of this data from other patient populations to the newborn infant. However, it does highlight consistent associations between the age of the PRBC pack and adverse patient outcome and therefore the potential for the PRBC pack to be biologically active in the critically unwell.

While in-vitro studies have consistently shown that older blood has the greatest potential to initiate immunomodulation[97, 146] it remains unknown if this is translated clinically. Recently it has been suggested that the immunomodulatory potential is dependent on the severity of illness the patient is experiencing at the time of transfusion exposure, similar to the two-hit hypothesis of TRIM [137]. It seems logical that sicker patients are far more likely to experience increased morbidities irrespective of PRBC exposure. In an attempt to control for underlying disease severity. This may explain why some studies show an increase in adverse outcomes while others show no difference. Gauvin et al, found that patients were more likely to experience storage associated adverse effects when their degree of illness severity was substantial [154]. However, Weinberg et al in a mild to moderately injured intensive care unit patient cohort, have reported that the receipt of blood stored beyond 2 weeks was independently associated with mortality, renal failure, and pneumonia. This data suggests that the deleterious effect of older blood on patient outcome may not be limited to the most unwell [152].

The potential for transfusion with "fresh blood" to reduce the incidence of adverse outcomes in the neonatal population was investigated in the ARIPI randomised controlled trial. In this study preterm infants weighing less than 1250 g received either a 'fresh' transfusion pack (<7 days old) or standard (2-42 days old) [155]. No difference in mortality or morbidity rates was observed between the 'fresh' or standard arms [155] suggesting no clinical benefit. However, the

average age of the PRBC packs in the standard arm was only 14 days meaning that many babies in the standard group also received fresh donor blood. Interpretation of the data is further confounded by a lack of a standard transfusion threshold between participating hospitals. This resulted in variability in the number of transfusions infants received between the study centres. This is an important consideration as it is already known that as the number of transfusions increase the chance of being exposed to immune inducing pathogens also increases [48].

While in vitro studies demonstrate biologically plausible immune effects in response to prolonged storage which could have the potential to effect morbidity and mortality [137], whether they are clinically significant remains open to question. As a result, any link between age of transfused red blood cells and adverse outcome still needs to be definitively answered. At best, it could be proposed that for patients with an underlying illness exposure to fresh blood may be beneficial, but once there is repeat transfusion exposure, any clinical benefit is lost.

2.3 Pre-Storage Leukodepletion

Due to the accumulation of immune-active molecules in RBCs with storage, attempts have been made to ameliorate these time-dependent processes. This has primarily centred upon pre-storage leukodepletion of RBCs which is currently the standard procedure in most hospitals worldwide [123]. This process involves filtering blood following collection to reduce the white blood cell (WBC) count by 99.9%. This processing step was developed on the premise that it would reduce transfusion-associated adverse outcomes by reducing immunomodulatory effects secondary to transfusion of leukocytes along with red blood cells.

Sparrow and colleagues investigated the effects of different PRBC preparations (length of storage time, untreated blood, buffy coat deplete or leukodepleted) on accumulation of immune-active components. In standard whole blood IL-8 accumulation continued to increase

as the age of the pack increased, whereas blood that was both WBC-reduced pre-storage and buffy coat-poor had undetectable levels of IL-8 [99]. Leukodepleted PRBCs did not demonstrate induction of CD11b expression previously shown to be an important marker of neutrophil priming and transfusion related immunomodulation [99, 156]. This is an important observation as primed neutrophils are thought to be a key component in the mechanism behind transfusion associated morbidities. When neutrophil priming occurs they are able to produce toxic oxygen metabolites ultimately resulting in a respiratory burst [156]. Cardo et al reported similar results with in vitro exposure to leukodepleted blood resulting in significantly lower CD11b expression and a failure to prime cultured neutrophils [156].

One of the first clinical studies to successfully demonstrate the benefit of leukodepletion was conducted by van de Watering et al [157]. Patients were transfused with standard buffy coat deplete packs, packs which underwent leukodepletion prior to storage or packs filtered following storage. Patients exposed to buffy coat deplete packs had significantly greater mortality rates than those exposed to the leukodepleted packs irrespective of the time of filtration [157]. Interestingly, some patients received a mixture of buffy coat and filtered packs. These patients still had a reduced mortality rate compared to those that exclusively received buffy coat deplete [157] suggesting that even limited exposure to leukodepleted blood improves outcome. If the two-hit hypothesis underlying TRIM holds, this finding would suggest that despite sensitisation of the immune system following exposure of the buffy coat deplete pack there is no activation of the immune system with subsequent exposure to leukodepleted PRBC. Ultimately, the most important risk factors for mortality appear to be the cumulative exposure and timing of exposure [157].

Similarly, Bilgin and colleagues found leukodepletion of PRBCs was associated with reduced mortality rates (pre-discharge and 90-day post-operative mortality) in adult patients

undergoing cardiac surgery [118]. However, in both standard and leukodepleted groups, as the number of transfusions increased mortality rates also increased supporting a dose response effect [118]. Rates of infection were also decreased with leukodepleted transfusions. Likewise, when leukodepletion was universally instigated in the Canadian healthcare system there was a significant overall decrease in mortality rates, fever and antibiotic use [158]. Initially the national Canadian healthcare study was designed to identify changes in infection rates. However, it also identified a strong association between exposures to buffy coat deplete blood and mortality rates, similar to that of Bilgn et al, with patients exposed to standard unfiltered RBC packs having two times higher mortality than the leukodepleted group [118, 158].

A common criticism of transfusion trials identifying mortality risk is their failure to prove a causal relationship between changes in clinical practice and the outcome of interest and the heterogeneous nature of the study participants. For instance, Watkins et al proposed that the reduction in mortality associated with leukodepletion could be as a result of a reduction in acute lung injury following a transfusion [159]. However, while respiratory distress was slightly less apparent for those exposed to the leukodepleted packed cells packs the overall incidence of Acute Lung Injury did not decrease [159]. To control for patient heterogeneity, investigators have attempted to stratify participants within their allocated study arms to ensure equal distributions of common baseline characteristics which could influence the study outcomes. While this has resulted in a lessening of the benefit of leukodepletion on adverse outcome the effect remains [160, 161].

Pre-storage leukodepletion of small volume pedi-packs used for neonatal transfusion results in undetectable levels of cytokines (TNF, IL-1, IL-6 and IL-10) within packs stored for 3, 21 and 42 days, used in a population of very preterm infants (n= 27 PRBC units) [162]. Furthermore, no difference was observed in circulating pro-inflammatory cytokines following transfusion with

leukodepleted PRBCs with the authors concluding that it was unlikely that pre-filtered RBCs result in cytokine changes in the recipient. This finding should be viewed with caution as patient sampling was discontinued early and samples were taken very early (1 hour post-transfusion), potentially missing any subsequent changes to cytokine levels. This is particularly important given that the cytokines sampled included early onset cytokines such as IL-1 and TNF which have an acute onset, whereas IL-6 and IL-10 show a later and more prolonged response potentially not peaking at the time point that the samples were taken [163]. In the largest randomised controlled trial investigating the potential beneficial effect of using leukodepleted PRBC transfusions in preterm infants, Fergusson et al. found that the group of infants, 247 infants born <1250 grams transfused with leukodepleted blood had a lower incidence of common neonatal morbidities such as ROP, BPD, and NEC, as well as a trend towards a reduction in bacteraemia and IVH [129].

However, leukodepletion may not completely abrogate the immunomodulatory effect of PRBC transfusion with implications for longer term clinical outcomes. Keir et al. reported that leukodepleted RBCs continue to have an immune-modulatory effect in this population of high-risk preterm infants. Pre- and post-transfusion samples were obtained from 28 preterm infants (<28 weeks' gestation) who received a transfusion of leukodepleted RBCs [63]. Following transfusion of leukodepleted PRBCs circulating levels of pro-inflammatory cytokine concentrations (IL-1ß, IL-8, TNF) and markers of endothelial activation (macrophage migration inhibitory factor and soluble intracellular adhesion molecule) increased in the neonatal circulation [63]. Similar findings were reported by Dani and colleagues with interferon gamma (IFNγ), IL-17 and MCP-1 remaining elevated for 48 hours post-transfusion [37]. Interestingly, these studies propose an additional mechanism, endothelial activation, which may contribute to the pathophysiologic processes that result in TRAGI and TRALI [164].

2.4 Repeated Transfusion Exposure

Numerous observational studies have observed a dose response effect with increasing transfusion exposure and/or cumulative volume of blood transfused associated with a greater risk of morbidity and mortality [165]. While this has led to a more restrictive approach to PRBC transfusion [47, 48], limiting both overall volume and donor exposure, the evidence supporting improved outcomes remains questionable [136].

Most studies investigating adverse immune responses to transfusion have focused on a single transfusion event [162]. Further, the majority of studies assessing the presence of a dose response effect have been retrospective analyses and, as such, have a number of methodological limitations. Bernard and colleagues investigated the association between repeated transfusion exposure, post-operative infection rate and a composite morbidity score [49]. The incidence of septic shock, pneumonia and a higher score on the composite morbidity rating scale increased not only after one transfusion but demonstrated a positive linear relationship with the number of transfusion units received [49]. A similar, cumulative response was observed by Santos and colleagues with intra-hospital death within the first month of coronary artery bypass in adults increasing by 77% with every additional transfusion [50] .

It could be argued that such a relationship is unsurprising with the increase in morbidity and mortality in fact reflecting greater physiological instability or number of co-morbidities in patients requiring multiple transfusions. However, when potential confounding factors are controlled for, such as surgery type, complexity and duration, mortality rates remain significantly higher following repeat PRBC transfusion exposure compared to those who did not receive a transfusion [49]. Additionally, patients who were originally considered morbidity free had an identical morbidity and mortality risk following multiple transfusion exposures [166].

While a "two-insult" hypothesis has been proposed to underlie TRIM, the exact mechanism/s causing this dose response effect remains poorly understood. It has been suggested that TRIM results in long term or permanent alteration to immune function [167] which is compounded by repeat transfusion exposure [50]. While the volume needed to induce TRIM is likely highly patient specific [49], numerous studies have demonstrated immunomodulation following exposure to 3 to 4 PRBC transfusions [49, 50, 166, 168]. While there is evidence for alterations in pro-inflammatory cytokine production and endothelial activation in response to single transfusion exposure [63], no studies have specifically investigated if repeat transfusion exposure results in similar adverse responses. There is, however, circumstantial evidence that repeat transfusion may indeed lead to endothelial activation and increased morbidity with an association between repeat PRBC transfusion exposure and increased ischemic damage reported in adult cardiac patients [168].

In the older paediatric population, oncology patients who received more than one transfusion with leukodepleted RBCs had lower survival rates than those exposed to only one transfusion [169]. Survival rates decreased further in those who received more than five transfusions [169]. Other than the randomised controlled trials of restrictive versus liberal transfusion thresholds [47, 48] and therefore, by default, repeat transfusion exposure in a significant proportion of the enrolled subjects, there is little data on the effect of repeat PRBC transfusions in the preterm population. Dos Santos et al have reported an independent dose response effect in response to transfusion exposure in the preterm, low birth weight population for morbidity and mortality risk [170]. Over a thousand preterm or low birth weight infants were enrolled in this study with 46% receiving their first transfusion within the first 28 days of life and over 30% subsequently receiving repeat transfusion exposures. If a transfusion occurred within the first 28 days of life, there was a 50% increased risk of mortality during the primary hospital admission and infants repeatedly transfused were two times more likely to die compared with non-transfused infants. While transfused infants were more likely to have had a 1- and 5-minute Apgar score <7,

respiratory distress syndrome (RDS), early and late onset sepsis, IVH and NEC [170], each of which are associated with poorer morbidity and mortality outcomes, transfusion remained an independent predictor of mortality when these factors were controlled for.

2.5 Washed Transfusions

Implementation of revised transfusion protocols that incorporate both leukodepletion and decreased storage time have not reduced the rate of transfusion-associated morbidities in preterm neonatal populations. This has led to the study of additional blood processing steps prior to transfusion, designed to remove any residual immune-modulatory agents. One such process is RBC washing where leukodepleted RBC packs are triple washed with 0.9% saline to remove the remaining microparticles, plasma proteins and antibodies [163]. Initial research has shown promising results, with adult and paediatric patients transfused with leukodepleted, washed RBCs having significantly less morbidity compared to those transfused with only leukodepleted packs [171].

RBC washing removes proteins, extracellular potassium, inflammatory cytokines and chemokines, as well as cell-free haemoglobin and RBC microparticles [172]. This substantial reduction occurs not only when packs were washed at the initial storage time, but also following storage for a number of days. Biffl and colleagues investigated the influence of prolonged storage of RBCs on the beneficial effects of washing [173]. Blood was collected from healthy adult volunteers with half undergoing leukodepletion on day one and the remainder left untreated. The transfusion packs were then stored for 42 days, with selected aliquots washed before isolation of the supernatant taken on days 1, 14, 21 and 42 of storage. As expected, with increasing storage time, the neutrophil priming ability increased irrespective of leukodepletion. However, when the packs reached the maximum storage time (42 days) in the post-storage washed samples there was a significant reduction in the neutrophil priming ability

of the RBC, with activity comparable to that seen with RBCs on the day of collection [173]. Furthermore, in vivo studies have shown that incubation of pulmonary endothelial cells with supernatant from washed RBC transfusion packs is not associated with increased endothelial permeability or pro-inflammatory cytokine and chemokine release that is observed following incubation with supernatant from standard RBCs [172, 174].

The evidence from clinical studies appears to support the beneficial effect of RBC washing on patient outcome. Cholette and colleagues investigated the effects of transfusing washed PRBCs on plasma cytokine concentrations in paediatric patients (<18 years of age) during cardiac surgery [163]. At 6- and 12-hours post-surgery the ratio of pro-inflammatory (IL-6) to anti-inflammatory (IL-10) cytokines was lower in patients transfused with washed versus unwashed PRBCs. While IL-6 concentrations were undetectable in both groups pre-operatively, the post-transfusion increase in IL-6, peaking at 6 hours, was lower in patients transfused with washed PRBCs. There is also clinical data supporting an effect of RBC washing on survival following a diagnosis of acute myeloid or lymphoid leukaemia [171]. Age of the pack and type of leukaemia were strong determining factors for predicting outcomes for patients >50 years of age [171]. This did not influence outcomes for younger patients. However, exposure to washed PRBCs was associated with significantly longer survival rates for patients <50 years of age. When a bone marrow or stem cell transplant and a transfusion occurred together, receipt of a washed pack significantly increased survival compared to exposure of leukodepleted PRBCs. While younger patients initially had a greater likelihood of attaining and prolonging remission compared to that of older patients, the survival rate of 75% could not be explained by a mere age effect and is proposed to be a benefit of receiving washed blood.

Not all evidence supports a beneficial effect of red blood cell washing. Wozniak and colleagues reported a reduction in RBC-derived microvesicles with mechanical washing but found no

difference in perioperative serum IL-8 values or concentrations of plasma RBC microvesicles, platelet and leukocyte activation, plasma cell-free haemoglobin, endothelial activation, or biomarkers of heart, lung, or kidney injury in adult patients undergoing cardiac surgery [175]. Further, the washing process itself may result in a decrease in haemoglobin and haematocrit and RBC damage and subsequent release of biologically active substances, including microparticles [172]. Some studies have shown that patients who have been exposed to washed PRBCs require significantly more transfusions than those who receive standard PRBCs [175, 176]. This has been proposed to be secondary to a reduction in red cell quality (up to 10-25%) [176]. Despite this, animal data suggests the washing process does not significantly or irrevocably damage RBC appearance or function. While there were increases in cell-free haemoglobin, it was estimated that only 0.5% of erythrocytes were damaged. As a result, inflammatory cytokine levels were significantly decreased, not only within the pack, but also in the transfused recipient [177].

Weisbach and colleagues investigated the effect of RBC washing on red cell quality in vitro and reported a significant reduction in free haemoglobin, 2, 3-DPG and an increase in lactate [176]. It is possible that these alterations could have been secondary to the fact that the RBC transfusion packs were stored at room temperature. This is known to result in a significant decrease in ATP, pH and 2, 3-DPG, important components in red blood cell integrity [178], and an increase in haemolysis [179]. The increase in microparticle number is thought to result from the additional filtration processes used to wash RBCs [172]. Using flow cytometry, antibodies to glycophorin A (GPA) and annexin V (AV) can be used to quantify RBC microparticles [172]. The washing process changes the phenotype of microparticles from GPA, AV positive to GPA intermediate, AV negative [172]. Although the proportion of GPA, AV positive was significantly decreased, it was surprising to see a change in microparticle phenotype [172]. While the importance or significance

of this is not known, it is possible the new phenotype could have a different immunomodulatory effect on the recipient's immune system.

It is evident that transfusion-related injury is not merely dependent on one factor, e.g. storage time or exposure, but a myriad of influences. While appropriately designed and powered randomised trials have been conducted in adult [171] and paediatrics patients [163], there is a pressing need to conduct studies investigating the effect of pre-washing RBCs in the preterm newborn population, who are heavily transfused and have significant rates of morbidity and mortality . The knowledge gap in the adult population has been recognised and is currently being addressed by the Washing of Allogeneic Red Blood Cells for the Prevention of Respiratory Complications (WAR-PRC) study (ClinicalTrials.gov Identifier: NCT02094118) [180]. However at the present time, there is insufficient evidence to support or refute the use of washed RBCs for transfusion in preterm infants to prevent morbidity or mortality [181]. It is hoped that this question may be definitively answered by the Transfusion with washed versus unwashed red blood cells to reduce morbidity and mortality in infants born less than 28 weeks' gestation: a multi-centre, blinded, parallel group, randomised controlled trial: (The WashT) study (Australian and New Zealand Clinical Trials Registry: ACTRN12613000237785) which is due to commence recruitment.

3 Physiological Response to Transfusion

While pre-clinical and emerging clinical data indicate an ability of RBC products to directly modulate immune cell function, a number of other pathophysiologic pathways have been postulated to contribute to the association of transfusion and subsequent adverse outcome. Anaemia itself, rather than transfusion exposure, may be the driving force behind the development of apparent transfusion-associated morbidities [182]. In particular, the degree of anaemia may be critical to the increased risk of specific morbidities such as NEC in the preterm

infant [65]. This is based on the clinical consequences of anaemia such as tachycardia, apnoea, and lactic acidosis, which have traditionally led to the clinical decision to transfuse. Furthermore, it is possible that the recognised major transfusion-related morbidities, particularly TRALI, TACO, and transfusion-associated dyspnoea represent exaggerated physiological responses to transfusion. As a result, a clear understanding of the normal response to progressive anaemia and the cardiovascular, respiratory and haemodynamic response to subsequent transfusion is critical.

3.1 Cardiovascular Responses to Packed Red Blood Cell Transfusion

A number of studies have documented compensatory haemodynamic changes in response to anaemia and subsequent transfusion. Fredrickson and colleagues found infants with a low haemoglobin (haematocrit ≤9) following transfusion demonstrated a significant improvement in cardiac output, 301 mL/min/kg pre-transfusion verses 253 mL/min/kg post-transfusion [120]. These changes were seen in conjunction with a decrease in lactate and increases in a number of measures representing oxygenation. In a population of sick, ventilated preterm infants, Cambonie and colleagues found that following a transfusion in the first postnatal week there was a significant increase in both stroke volume and left ventricular output (LVO) [183].

Alkalay and colleagues investigated the effects of progressive anaemia and its impact on the cardio-respiratory system. In a group of preterm infants considered “stable”, extubated and with no evidence of a patent ductus arteriosus, tachycardia, tachypnoea or apnoea, transfusion resulted in an increase in systolic and diastolic blood pressure, but contrary to the Cambonie study, no change in left ventricular function or stroke volume [119]. However, on closer inspection of the data, those infants with the lowest haematocrit exhibited a larger increase in cardiac output with a significantly greater left ventricular end diastolic and systolic diameter in addition to increased stroke volume [119]. Interestingly, in those infants with higher haematocrits, transfusion was associated with subsequent tachypnoea, suggesting the potential for adverse

cardio-respiratory effects following transfusion. It remains unclear if this may be due to alterations in pulmonary blood flow following transfusion or inflammatory processes as seen in TRALI secondary to TRIM.

As previously stated, a transfusion is given in order to correct the consequences of anaemia, such as tachycardia, tachypnoea and apnoea. Therefore, theoretically there should be a decrease in cardiac output due to an increase in haemoglobin and therefore oxygen delivery. Kanmaz and colleagues studied 35 'stable' preterm infants who were less than 35 weeks' gestation. Following a 15mL/kg leukodepleted PRBC transfusion there was a significant decrease in cardiac output and lactate and an increase in perfusion index [184]. However, the degree of anaemia (determined by haematocrit) correlated poorly with the post-transfusion changes in cardiac function. Similar findings are reported by Yu and colleagues with significant decreases in cardiac output and stroke volume following a PRBC transfusion, but no change in heart rate or blood pressure noted [185].

3.2 Cerebrovascular Changes and Red Blood Cell Transfusion

Fluctuations in systemic and cerebral blood flow have been associated with the development of IVH in the preterm infant [186]. Therefore, it is possible that PRBC transfusion-associated changes in haemodynamic responses may have a detrimental effect on the developing cerebrovascular system primarily due to immaturity of cerebral autoregulation [186]. Nelle and colleagues found that following a transfusion there was a significant decrease in cardiac output and stroke volume [187]. While blood flow velocities decreased significantly in the anterior cerebral artery and the internal carotid artery, red cell transport in the internal carotid artery but not the anterior cerebral artery rose. Assuming that the blood flow velocities in cerebral arteries reflect the actual blood flows, this suggests that the overall oxygen transport to the brain is improved by transfusion, but with marked regional variations [187].

These findings have been replicated by a number of studies. In a small study of 23 transfused preterm infants. Quante et al described initially high cardiac output and anterior cerebral artery blood flow pre-transfusion which both fell and remained significantly lower up to 72 hours post transfusion [188]. Similarly, Ramaekers and colleagues showed that cerebral blood flow velocity in stable preterm infants decreases with transfusion in accordance with the increase in oxygen supply [189]. This has been confirmed by studies employing Near Infra-Red Spectroscopy (NIRS) and Cerebral Doppler Ultrasonography that confirm that blood transfusion related improvements in cerebral oxygen supply occur hand-in-hand with decreases in cerebral blood flow velocity [190-192].

3.3 Peripheral Haemodynamic Response and Red Blood Cell Transfusion

While most attention has been given to cardiovascular and cerebro-vascular responses to packed red blood cell transfusion, transfusion also impacts upon the perfusion and oxygenation of other tissues. Perhaps the most pertinent in the preterm infant are transfusion related alterations in blood flow to the gut which are proposed to be a contributary factor in the development of NEC [193]. Intriguingly, the cerebro-splanchnic oxygenation ratio where near infra-red spectroscopy derived tissue oxygenation of the cerebral and splanchnic circulations is compared, decreases in NEC, consistent with independent regulation of the gut and cerebral vascular beds [194]. Is it proposed that the splanchnic circulation in the preterm infant is less adept to vascular regulation [194], therefore a transfusion can lead to variability in perfusion and oxygen delivery [194].

Preterm infants receiving a blood transfusion in the presence of a significant Patent Ductus Arteriosus (PDA) show a reduction in mesenteric flow 4 hours after transfusion, an effect proportional to the volume of a PRBC transfusion [193]. However, even in the absence of a clinically significant PDA, transfusions per se are associated with increases in viscosity and mesenteric oxygenation, and a lower fractional extraction of oxygen as determined by NIRS

measurement of gut regional tissue oxygenation [190, 195]. However, evidence for changes in mesenteric vessel mean flow velocity is more contentious with some studies in stable preterm infants showing no change in splanchnic and mesenteric arterial blood velocity [196].

4 Role of the Sex of Transfusion Donor

While characteristics of the donor have been extensively investigated in the solid organ donation literature, characteristics of blood donors has been relatively under researched. This is despite a blood donation having the potential to exert a similar immunological effect in the recipient to that of organ donation [197]. Emerging evidence suggests that blood donor characteristics may indeed influence a recipient's response, although the biological mechanisms underpinning this response remain elusive [198]. While the majority of research to date has focused on plasma and the morbidities associated with plasma exposure, there is increasing awareness that exposure to red blood cells poses a similar risk [199]. In addition, the majority of existing studies in the literature are observational and retrospective, meaning the risk of bias is high and the interpretation of results limited.

4.1 Human Leukocyte Antibodies

In 1983, Popovsky and colleagues described a condition in which patients experienced respiratory complications following a transfusion [200]. These complications were undistinguishable from the adult presentation of acute respiratory distress syndrome [200] and consequently the term TRALI was coined. They identified that 90 percent of patients thought to meet a TRALI diagnosis had been exposed to blood products from a donor containing antibodies reacting to either Human Leukocyte Antigen (HLA) or Human Neutrophil Antigen (HNA) in the transfusion serum [201]. While the exact pathogenesis behind TRALI development is still unclear, HLA antibody involvement has been as a proposed mechanism, particularly for blood products from a from a female donor where the induction of anti-fetal antibodies occur during pregnancy [202].

HLA molecules are part of the human major histocompatibility system (MHC) and are a family of cell surface glycoproteins that bind to intracellular and extracellular peptides [203]. The HLA family is located on chromosome 6, which contains hundreds of genes, including 40 of which encode leukocyte antigens [204]. The HLA family is further separated into two distinct functional and structural groups; HLA Class I and HLA Class II [205]. Class I HLA antigens are carried in varying degrees on almost all nucleated cells. Class II antigens are carried on immune cells such as activated T cells, B cells, macrophages, dendritic cells, cells of the thymus with other cells are able to express Class II molecules through exposure to interferon-γ [204]. The HLA molecule is critical for the induction and regulation of immune responses [205]. In a normal immune response circulating T-cells recognise and interact with foreign HLA molecules, resulting in T-cell activation. Through this activation there is a significant increase in T-cell number and consequently a release of cytokines resulting in an immune response [205]. The mechanisms in which the two HLA classes work are different. HLA Class I molecules directly present invading viruses to CD8 T-cells which destroy the infected cell [205]. HLA Class II molecules present exogenous peptides to CD4 T-cells, which stimulate a widespread immune response [205].

In the setting of an inappropriate immune response, such as with organ transplant or transfusion, the immune response produced by the HLA molecules is rapid and extremely robust [206]. The HLA response is thought to be so powerful due to a high degree of polymorphism increasing the possibility of transfusing incompatible products or tissues [207] and the HLA system's ability to not only elicit an immune response directly but also indirectly [204]. This is thought to occur through HLA molecules circulating through the spleen allowing splenic macrophages and dendritic cells to stimulate the recipient's adaptive immune response [205]. An aberrant HLA response has been identified in a number of autoimmune conditions as well as defective HLA gene encoding being partly responsible for conditions such as narcolepsy and

haemochromatosis [208]. However, the most widely researched area is transplant medicine [206, 208] where donor specific HLA antibodies have been linked to transplant injury [209].

How HLA antibodies cause a transfusion reaction is not fully understood for all transfusion associated conditions. In the case of TRALI, it is thought that anti-HLA or HNA antibodies bind and stimulate circulating leukocytes, neutrophils and other cytotoxic cells which subsequently release reactive oxygen species resulting in pulmonary microvascular endothelial damage [210]. This initiates an inflammatory response in the lungs, causing an increase in oedema and fluid in the alveoli and the respiratory complications likened with TRALI [206]. However, not all transfused patients go on to develop antibody-mediated complications with the individual's predisposition being the greatest determining factor [210]. In the setting of TRALI, this may be trauma or an inflammatory condition involving the lung. A threshold model has also been proposed with the HLA antibodies ability to prime cells of the inflammatory system reliant on the patient's predisposition. This then dictates the oxygen sufficiency and oxygen requirement and in turn the severity of TRALI [210]. Both HLA Class I antibodies and HLA Class II antibodies have been linked with development of TRALI. However, Class I antibodies are thought to pose the greatest mortality risk with one study finding that 62% of mortality cases were associated with HLA Class I antibodies with Class II antibodies accounting for just 11% [211].

To date, platelets and plasma products have been the key blood products thought to have a role in antibody-mediated pathophysiological processes [212]. With platelets the HLA molecule on the platelets' surface is readily detached during storage [205]. Therefore, over the course of storage, a significant number of HLA antigens are shed and the transfused patient potentially exposed to a high dose of donor HLA antigens [205]. Universal leukodepletion and storage time limits were thought to be the solution to the risk posed by HLA [207]. However, this has not been the case with HLA antigen still detected in leukodepleted transfused packs [207]. In contrast, HLA

Class I is present at low levels or absent from erythrocytes [205]. Therefore, the role of RBCs in antibody-mediated transfusion reactions has been largely overlooked. However, a single RBC can carry around 100 to 2000 Class I HLA and with 1000 times more red blood cells to leukocytes in a leukodepleted PRBC transfusion pack, the HLA content may still be as large [212].

4.2 Sex of Donor and Plasma Transfusions

Historically, the greatest risk posed by transfusion has been the potential for the transmission of viral infections, yet with considerable research and technical advances focussed on minimising this risk, the risk of transfusion related viral infections is now extremely low [202]. In fact, blood product transfusions are now the safest they have ever been [213]. As a result, transfusion related inflammatory conditions are now of greatest concern. One of these inflammatory conditions is TRALI which has become one of the leading transfusion related morbidities [202]. In the early 90's rates of TRALI were worryingly high and therefore the Serious Hazards of Transfusion (SHOT) group in the United Kingdom conducted the largest study focusing on a ten-year period specifically looking at the incidence of TRALI, leukocyte antibodies and proposed risk reduction steps. Chapman and colleagues investigated the clinical and practical implementations of a male donor only plasma strategy and found that simply through adoption of a male only donor strategy a 79% reduction in highly likely or probable TRALI cases could be achieved [202]. However, this reduction was only seen for patients exposed to fresh frozen plasma (FFP) and platelets (PLTs). Interestingly, FFP was almost 7 times more likely to be associated with a TRALI diagnosis than RBCs and PLTs were 8 times more likely than RBCs [202]. In addition, and consistent with very early work [214, 215], was the significant number of antibody positive female donors associated with all TRALI episodes, with 65% having an identified antibody type [202]. Interestingly, HLA Class II accounted for 61% of antibodies detected [202]. This is despite HLA Class II antibody testing only beginning 6 years into the study.

The high levels of HLA Class II antibodies is contradictory to previous research, showing an increased association between TRALI and the presence of HLA Class I antigens rather than HLA Class II [211]. A role for HLA Class II antibodies in TRALI has previously been questioned, as they are not carried on neutrophils [216], the immune cell thought to be central to the pathogenesis of this condition [210]. Reil and colleagues tested over five thousand donors to specify HLA and HNA type and linked this with an acute respiratory event and reported similar findings to the SHOT study. While HLA Class I antibodies represented 73% of the antibodies detected, only 19% were associated with an acute respiratory event [217]. The authors postulated that while HLA Class I antibodies were more common, they are less potent [217]. Interestingly, when all respiratory events were investigated, they found that over 81% of cases were associated with HLA Class II and HNA antibodies, despite these representing only 36% of the overall antibodies detected in the donor pool [217]. This may be the result of a secondary mechanism through which HLA Class II antibodies can induce neutrophils [218]. It is thought that HLA Class II antibodies may activate accumulated neutrophils in the pulmonary microvasculature by binding to monocytes and consequently releasing pro-inflammatory cytokines [218] explaining the strong association between the less frequently expressed Class II antibody and post-transfusion respiratory deterioration.

Despite the significant reduction in the incidence of TRALI following the adoption of the male only donor plasma strategy, a number of questions remain. In Australia, the most recent data from the National Blood Authority of Australia reports rates of TRALI of 1.4%, suggesting a high level of underdiagnoses, particularly compared to levels reported internationally [219]. The SHOT study relied on clinician awareness of the research studies aim when reporting whether patients met the current guidelines at the time, introducing an element of bias from both under- and over-reporting [202]. Therefore, the 79% reduction in TRALI cases may not be an accurate representation. Further, the effect of multiple transfusions and a mix of transfusion

types was not taken into account. This is likely to be important with data for PRBC transfusion, for instance, reporting a significantly greater mortality risk in patients exposed to multiple transfusions (3 or more) even following adjustment for confounding variables [49, 166].

Tynell and colleagues, investigating short-term mortality rates after plasma transfusion in adults specifically addressed the issues of donor sex and multiple transfusion exposure [220]. In a retrospective analysis of almost 100,000 patients in 30 Swedish hospitals, they found that it was not until patients were exposed to 3 or more transfusions that there was an increase in mortality. Further, 14-day mortality rates were higher in those patients who received plasma from female donors compared to those who received plasma from males (8.43% versus 7.5%). After controlling for potential confounding variables, the relative risk of mortality increased in those exposed to 5 or more plasma transfusions compared to 3-4 (1.32 (CI1.17-1.49) versus 1.16 (CI 1.06-1.27). While the influence of disease severity remains an important consideration, they concluded that transfusion of plasma from female donors confers a short-term survival disadvantage, an effect that appeared dose dependent [220]. Eder and colleagues evaluated the implementation of a similar male donor only policy throughout the American Red Cross in a heterogenous population of oncology, cardiac and infectious disease patients with a particular focus on mortality [215]. Although the study design again raises the possibility of selection bias, the data from this study is consistent, with over 71% of patients with TRALI who subsequently died receiving plasma from an antibody positive female [215].

Very few studies have looked at the effects of mixed transfusions, an element that we know may change or suggest the overall severity of the patient's clinical state. Gajic and colleagues investigated the effect of exposure to 3 or more transfusion components (platelet and/or plasma) from either male or female donors on respiratory instability. While rates of lung injury and circulatory overload were similar between the two groups, patients exposed to three or

more male donor packs were more likely to have fewer ventilated days [221]. Further, in patients that were exposed to a transfusion product from a female donor, the PaO_2/FiO_2 ratio worsened significantly [221]. PaO_2/FiO_2 ratio is commonly used as a proxy measure for lung injury in the adult and paediatric populations and has been shown to have a strong relationship with disease severity and mortality risk [222]. While the authors concluded that high volume transfusions of FFP and PLTs seemed to be more clinically important than transfusions of RBCs, almost 95% of patients exposed to FFP or PLTs also require a PRBC transfusion [223].

Interestingly, all studies observed a significant increase in the number of deaths in patients who were exposed to a multiparous woman's blood. This is primarily thought to be due to the generation of anti-fetal antibodies in pregnancy. The only prospective double blinded randomised trial in donor sex research investigated whether using blood from a female who had previously been pregnant resulted in both an inflammatory response (characterised by circulating cytokines) and clinically significant sequelae. Patients requiring at least 2 transfusions of plasma were eligible and these patients either received blood from a 'non-immunised' donor (never pregnant or transfused) or 'immunised' donor (previously had 3 or more live births). TNF concentrations significantly increased following transfusion exposure irrespective of whether they received plasma from an immunised or non-immunised donor [224]. However, the increase in TNF was far more pronounced in those who received plasma from immunised donors. In addition, patients in this study arm had a significant decrease in their PaO_2/FiO_2 ratio, whereas mean blood pressure (MBP) significantly increased for patients in the non-immunised study arm [224]. The authors proposed that together, the changes in PaO_2/FiO_2 and TNF concentration represented impairment and severity of lung injury. What mechanism/s underlie the association between adverse outcome and repeated exposure to blood products from multiparous females currently remains unknown.

The retrospective nature of donor gender studies does mean, as a result of inherent bias, they are methodologically flawed. It has been suggested that the true effect of female donor exposure will only be definitively worked out through a randomised trial. Interestingly, many patients exposed to transfusion packs from a female donor do not exhibit any negative consequences. Therefore, it is highly likely to be patient specific and related to the proposed two-hit hypothesis or threshold model. It is clear that universal leukodepletion has not accomplished prevention of antibody mediated TRALI [207] and that TRALI is not limited to exposure to plasma and platelet transfusions. All studies have failed to acknowledge the role that red blood cells may play in the donor effect with most concluding that the evidence for red cells is not strong enough to justify any need for change in red cell practice. While it was previously thought that 50% or more of plasma is required to illicit a TRALI response it is now hypothesised that less than 10% of plasma may be needed [225]. In fact, of the TRALI cases reported to the Canadian blood service between 1998 and 2004, 31% were implicated with PRBCs, 29% with PLTs and 18% with FFP [225]. Taking into account the extremely low residual plasma in an PRBC transfusion, this highlights the danger that a PRBC transfusion may pose to the patient.

4.3 Sex of Donor and Red Blood Cell Transfusions

Red blood cell transfusions are one of the most common medical interventions with more than 100 million red cell packs collected and transfused worldwide each year [197]. Yet the RBC has been largely discounted as a contributor to HLA sensitisation due to the low levels carried on RBCs and RBC pack alterations such as leukodepletion. The adult literature specifically focusing on PRBC transfusion is conflicting with most concluding there is no effect of donor sex on the incidence of transfusion associated morbidities [226, 227]. However, Chasse and colleagues have reported an association between donor sex and cumulative exposure and survival after PRBC transfusion. The transfusion of each additional PRBC unit from a female donor was associated with an increased risk of death of 8% compared with receipt from a male donor (adjusted HR,

1.08; 95% CI, 1.06-1.09 for each additional unit transfused; P < 0.001). They found that female sex was associated with reduced survival in both male recipients (adjusted HR, 1.08; 95% CI, 1.07-1.10 for each additional unit transfused; P <0.001) and female recipients (adjusted HR, 1.03; 95%CI, 1.02-1.05 for each additional unit transfused; P < .001).

However, in a retrospective study of 9907 patients who had undergone coronary artery bypass grafting or aortic valve replacement surgery, Bjursten and colleagues investigated a range of transfusion related risk factors including the age of the blood product, sex of the donor, sex matching of the donor and recipient, as well as post-transfusion treatment [228]. While they found a significant association between mortality and transfusion of 1-2 units of non-leukodepleted PRBCs they also found an excess of mortality for transfusions of sex mismatched PRBCs but not specifically transfusion recipients receiving blood from female donors [228].

Importantly, a meta-analysis investigating the associations between donor characteristics and short- and long-term transfusion outcomes for the transfused recipient has re-visited the potential role of donor sex [229]. From the 59 studies that met the eligibility criteria (50 observational, 9 interventional), 17 donor characteristics where identified that influenced transfusion outcome. Donor sex demonstrated associations with reduced survival at 90 days and 6 months in male recipients who receive donated blood from females (hazard ratio 2.60 [1.09, 6.20] and hazard ratio 2.40 [1.10, 5.24], respectively) [229]. However, the risk of bias and confounding of all the included studies was high and the quality of evidence was graded as very low to low.

There are numerous studies looking at potential inherent biological differences between male and female red cells [230-232]. In vitro studies have shown that male RBCs have a greater level of haemolysis, a common characteristic of storage lesion, which is considered a potential

contributor to transfusion associated morbidities [230]. Meanwhile, RBCs from females show greater reductions in acetylcholinesterase enzyme activity and subsequent increased membrane rigidity following exposure to adrenaline [231]. Whether sex-specific differences in the RBC themselves are of clinical importance and contribute to a sex-specific risk of transfusion related adverse outcome remains unknown.

As previously stated, HLA sensitisation occurs following organ transplantation, pregnancy and previous blood transfusions [212]. The complexity of HLA sensitisation and subsequent interaction is often overlooked for a far more simplified, however misconceived view of the RBCs' role. Approximately 20% of multiparous women have circulating HLA antibodies, therefore a woman of child bearing age may pose the greatest risk to the transfused recipient [207]. While no difference in transfusion related mortality is observed between recipients who receive blood from female donors with no history of pregnancy compared to from a male donor, the risk significantly increased for all male patients transfused with RBCs from a female donor with any history of pregnancy [233]. Importantly, this effect was enduring, with no limit to the time from pregnancy to transfusion and adverse outcome seen. Interestingly, this association was also influenced by the age of the transfusion recipient. The strongest association between transfusion with PRBCs from a previously pregnant female donor and mortality was seen for male patients 0 – 17 years of age (n= 2534) who received 2 or more transfusions. In fact, the youngest males were almost two times more likely to die if they received PRBCs from an ever-pregnant female donor [233]. However, this effect only occurred if all transfusions were from previously pregnant female donors.

There is currently only a single study that has focused solely on the potential for donor sex specific effects in a preterm population. Murphy and colleagues looked at 170 extremely preterm infants who received a transfusion from either exclusively male or a mixture of male

and female donor PRBCs. Infants who received any PRBC transfusions from a female donor had significantly higher rates of BPD, SIP/NEC and any morbidity or death. In addition, these infants also had significantly longer length of NICU stay [234]. This group were, importantly, also exposed to a greater number of transfusions overall. When this was controlled for the effect of donor sex was lost, with only increasing transfusion exposure associated with BPD, any morbidity, and length of stay [234]. However, subset regression analysis comparing PRBC transfusion from exclusively female donors with transfusion with male donor blood, demonstrated a significant interaction between female donor blood and number of transfusions for any morbidity. This is consistent with the result from the study by Chasse and colleagues where the odds of any morbidity increased when additional transfusions were from a female donor [234]. The authors concluded that while their data appears to suggest that donor sex impacts the preterm infant's response to transfusion, large prospective clinical trials are needed to determine not only the significance of the associations, but also the likely causal pathways involved.

While contentious, these results seem to suggest that the greatest risk to the transfused population is exposure to blood from a female who has been pregnant. This has vast consequences for the donation community and in particular, the preterm population. It is clear that the challenge for transfusion medicine is to further minimise the risk of transfusion-associated morbidities, while maintaining a healthy balance between those eligible and ineligible for the donor pool. While other common medical interventions such as pharmaceuticals are highly regulated and have almost no product variation, each blood donor and blood product are biologically different. The blood donor is therefore the greatest source of variability and still the least regulated step, with little likelihood of achieving standardisation of blood products [235].

5 Summary

Although transfusion of packed red blood cells saves lives in the neonatal intensive care setting, there is increasing awareness that transfusion is an independent predictor of adverse outcome. Whether these associations are a response to RBCs themselves, the time-dependent accumulation of bioactive substances in the supernatant, or both, remains unknown. A growing body of data supports a potentially detrimental pro-inflammatory response to transfusion, resulting in endothelial activation in the extremely preterm infant, though these studies have focused on a single transfusion exposure during the convalescent period weeks after birth [37, 63]. However, extremely preterm infants are a particularly heavily transfused patient group, receiving on average 3-5 blood transfusions often early in their admission to NICU [48].

Evidence in other critically ill populations supports significant benefit from modifications in blood product processing including RBC washing. This has never been studied in the preterm infant. Therefore, this thesis will compare the response to repeat transfusion exposure with standard unwashed leukodepleted PRBCs to transfusion with washed leukodepleted PRBCs in the extremely preterm infant. Specifically, I will focus on two potential pathways through which transfusion exposure may be related to adverse neonatal outcome. I will investigate if transfusion with washed packed RBCs results in a reduced transfusion-related inflammatory response. I will also investigate whether the acute physiological response to transfusion, in particular changes in cardio-respiratory stability, differs between those transfused with unwashed versus washed PRBCs and whether these physiological responses are influenced by transfusion-related immunomodulation. Finally, I will investigate if the sex of the PRBC donor confers additional benefit to the transfusion recipient, further reducing the risk of adverse consequence in those infants who receive washed PRBCs.

Chapter 2

Background, Hypothesis and Aims

2.1 Background

Extremely low gestational age infants are at a greater risk of poor outcome than any other population, thereby incurring substantial societal and economic cost. As such any reduction in mortality in combination with improvement in survival free of significant neonatal morbidity would be of substantial short and long-term benefit. Emerging evidence in other critically ill populations supports significant benefit from modifications in blood product processing including RBC washing, though this has never been studied in the preterm infant. There is increasing awareness that transfusion of blood products may be an independent predictor of adverse outcome with the incidence and severity of major neonatal morbidities including BPD, IVH, ROP and NEC, associated with the number and volume of PRBC transfusions [7]. Limitations in the existing data, including retrospective study design and small sample size, mean there is a pressing need for large studies to be conducted prospectively to address ongoing uncertainty about the safety of transfusion in this heavily transfused population.

Whether these associations are a response to red blood cells themselves, the time-dependent accumulation of bioactive substances in the supernatant (storage lesion), or both [100], remains unknown. This thesis will determine the potential for transfusion with washed PRBCs to modify the transfusion recipient's immune response to transfusion exposure. This will be a critical first step which will inform large scale clinical studies powered to determine if this intervention reduces the incidence of transfusion associated neonatal mortality and increase survival free of significant neonatal morbidity when compared to current standard PRBC transfusion practice. As such, data from this thesis will contribute to bridging the gap between research regarding transfusion related adverse outcome and translation into practice. The study outcomes will likely have enormous clinical benefit to the extremely preterm infant in whom transfusion is almost unavoidable and inflammation is a common pathway to life-long injury.

2.2 Aims

The study will examine the potential benefit of using washed leukodepleted red blood cells as an alternate transfusion product for extremely preterm infants.

1. To determine the effect of pre-transfusion washing of allogeneic red blood cells on end organ physiologic stability in preterm infants <29 weeks' gestation receiving a transfusion.
2. To determine whether transfusion of washed leukodepleted red blood cells results in a reduction in post-transfusion levels of pro-inflammatory cytokines and markers of endothelial activation.
3. To establish the effect of pre-transfusion allogeneic red blood cell washing on donor sex related immune responses in the transfusion recipient.

2.3 Hypotheses

Based on previous research, I hypothesise that preterm infants <29 weeks' gestation transfused with washed red blood cells will exhibit a reduced pro-inflammatory response compared to those transfused with unwashed red blood cells.

1. Washed compared with unwashed leukodepleted allogeneic red blood cell transfusion will attenuate adverse physiological end organ responses in the preterm infant.
2. Washed compared with unwashed leukodepleted allogeneic red blood cell transfusion will attenuate inflammatory and endothelial responses in the preterm infant
3. Washed compared with unwashed leukodepleted allogeneic red blood cell transfusion will attenuate donor sex related effects on immune responses in the transfusion recipient, specifically those in response to exposure to red blood cells from a female donor.

Chapter 3

Methods

3.2 Participant Recruitment and Randomisation

3.2.1 Participant Recruitment

Transfusion naïve extremely preterm infants born less than 29 weeks at the Women's and Children's Hospital, Adelaide, Australia and Flinders Medical Centre Neonatal Unit, Adelaide, Australia were eligible for study enrolment. Potential families were screened and approached at the time of delivery or prior to the newborns' first elective transfusion. Infants with major congenital abnormalities or those infants who had already received an elective transfusion were excluded from recruitment. All parents gave written, informed consent for their newborns' participation (Appendix 1.1 & Appendix 1.2). The study was approved by The Australian Red Cross Blood Service Human Research Ethics Committee (ethics approval number 2013#06) (Appendix 2.1) and the Women's and Children's Hospital Human Research Ethics Committee (ethics approval number REC 2498/9/15, HREC/12/WCHN/5, SSA/12/WCHN/56, SSA/17/SAC/230) (Appendix 3.2) with site-specific approval obtained from Southern Adelaide Local Health Network. An amended ethics submission was approved by the Australian Red Cross to obtain the sex of the transfusion donor (ethics approval number 2013#06) (Appendix 3.3).

3.2.2 Haemoglobin Trigger for Transfusion

The decision to transfuse was based on a pre-defined restrictive haemoglobin threshold as per the Premature Infants in Need of Transfusion (PINT) study (Table 3.1)[48]. Haemoglobin levels were determined by complete blood picture for each infant.

Table 3.1 Transfusion algorithm based upon modified transfusion threshold employed in the PINT study

Age (days)	Blood sampling	Respiratory support*	No respiratory support*
1-7	Capillary	≤135	≤120
	Central	≤122	≤109
8-14	Capillary	≤120	≤100
	Central	≤109	≤90
≥15	Capillary	≤100	≤85
	Central	≤90	≤77

*Hb threshold levels (g/L)

3.2.3 Subject Randomisation

Subject randomisation for both study sites was undertaken by the on-call transfusion laboratory technician. Transfusion laboratory staff were unblinded to red cell pack type allocation in line with Australian Red Cross Blood Service guidelines for safe provision of RBCs for transfusion. A computer-generated randomisation schedule using balanced variable block design of 6 was generated with infants randomised into unwashed or washed groups with an allocation ratio of 1:1. Within each study arm, infants were stratified by gestational age, 23^{+0} to 25^{+6} and 26^{+0} to 28^{+6} weeks. For any additional transfusions an infant required, RBC pack type consistent with the original allocation was issued. Infants were transfused according to standard nursery practice, fasting for 4 hours prior, for the duration of, and for 4 hours after the transfusion. At WCH site fasted infants received either total parenteral nutrition or for those who had already reached full enteral feeds prior to fasting, intravenous 10% dextrose. At the FMC site, infants requiring a transfusion were not fasted. The contribution of enteral feeding during transfusion to significant morbidity, specifically NEC, is debated and is the subject of the ongoing WHEAT randomised control trial [236]. All transfusions were a fixed volume of 15mL/kg, transfused over 3 hours via a peripheral intravenous cannula.

3.2.4 Red Blood Cell Manufacture, Blinding and Storage

All RBCs were leukodepleted, O Rhesus (Rh) negative. Only O Rh negative packs were only used to limit pack wastage and was standard practice at the study sites. Three day old,

leukodepleted, O Rh negative, RBC packs were washed by the Red Cross Blood Service. This involved three consecutive washing steps with 200mL 0.9% saline following collection and prior to storage. At each wash, saline was removed from packs by centrifugation at 2900 x g for 5 minutes (wash 1 and 2) and 5000 x g for 5 minutes (wash 3). Red cells were then resuspended in 100mL Saline Adenine Glucose Mannitol (SAG-M) and divided into 4 paediatric packs and stored at 2 – 6˚C for a maximum of 14 days. For both unwashed and washed packs, storage was limited to 14 days to control for 'storage lesion'. Blood products required for the study (4 unwashed leukodepleted packs and 4 washed leukodepleted packs) were obtained weekly from The Australian Red Cross Blood Service in Victoria and the order adjusted according to demand if needed. A washed pack was retained at the distributing site for quality control purposes. All packs leukodepleted and washed remained at the central WCH site and were issued to both neonatal nurseries by the WCH transfusion laboratory.

Study investigators, attending clinicians and nursing staff and parents were blinded to product allocation through the use of an opaque sticker applied to the transfusion pack by the transfusion laboratory. This alteration was approved by the Australian Red Cross Ethics Committee (ID2013#6). Transfusion pack issue and administration complied with all other current ordering and safety protocols. Compliance with the randomised intervention was managed by the transfusion laboratory with each study participant and allocated product identified electronically.

3.3 Clinical Data Collection

3.3.1 Plasma Collection

Infants had 0.4mL venous or peripheral capillary whole blood samples collected in lithium heparin tubes immediately prior to and 4-6 hours following the completion of the transfusion. A 1mL sample was also collected from the RBC pack. All samples were centrifuged immediately

at 3500 x g for 5 minutes and with the plasma aliquot stored at -20°C for batched cytokine analysis.

3.3.2 Cardio-Respiratory Data Collection

In order to understand and determine the normal physiological responses to progressive anaemia and the hemodynamic, cardiovascular and respiratory responses to a transfusion, a number of physiological parameters were collected at the primary transfusion exposure. Data immediately prior to the transfusion, throughout and then up to 10 hours following their transfusion exposure was collected retrospectively from the subject's medical record (Tables 3.2, 3.3, 3.4). Systolic (SBP), diastolic (DBP) and mean arterial blood pressure (MBP) was measured invasively from an umbilical arterial catheter or peripheral arterial catheter, when present, or by non-invasive blood pressure measurement. Heart rate (HR) and respiratory rate (RR) were measured using ECG electrodes and the impedance pneumography waveform from the patients electronic monitoring (Phillips 600X monitoring system). Percentage time out with saturation target band was calculated from an hourly histogram using in-bedded software on the Phillips monitors. For ventilated neonates, mean airway pressure and fractional inspired oxygen was derived from the ventilator (Drager Infiniti C500) while for those infants on non-invasive respiratory support (BCPAP or Optiflow) the set end-expiratory pressure and inspired oxygen was recorded. Collection of this data allowed determination of whether preterm infants exhibited signs of Neonatal transfusion associated circulatory overload (nTACO), Neonatal transfusion associated lung injury (nTRALI) and Neonatal transfusion associated dyspnea (nTAD), not previously identified in the preterm infant.

In 2011, the International Haemovigilance Network proposed a set of standardised definitions for non-infectious transfusion reactions including a range of hemolytic reactions and non-hemolytic transfusion definitions [71]. As these definitions were specifically designed for the adult patients, in 2018 the NHS Blood and Transplant agency adapted the non-hemolytic definitions

specifically for neonates. These definitions are the first of their kind for the neonatal population and were used as a guide for measuring the cardio-respiratory function of the current extremely preterm population. nTACO was defined as occurring within 6 hours post transfusion and the patient exhibiting 4 or more of the 5 conditions listed below (Table 3.5). nTRALI was defined as no evidence of positive fluid balance and evidence of acute respiratory distress (ARDS) for up to 6 hours post transfusion and bilateral pulmonary infiltrates on x-ray up to 10 hours post transfusion. nTAD was defined as occurring up to 28 hours following transfusion exposure and displaying ARDS but no evidence of nTACO or nTRALI. In addition to these definitions, a respiratory deterioration was defined as a sustained increase in fraction of inspired oxygen (FiO_2) or any increase in MBP or a change in ventilator mode, initiated by a worsening of respiratory illness severity.

Table 3.2 Haemodynamic parameters

Prior to Transfusion	Post Transfusion
Haemoglobin (g/L)	Haemoglobin (g/L)
Pack cell volume (L/L)	Pack cell volume (L/L)
Lactate (mmol/L)	Lactate (mmol/L)

Table 3.3 Cardiovascular Parameters

1-4 hours prior to transfusion	During transfusion	1-4 hours post transfusion	5-10 hours post transfusion
Mean arterial pressure	Mean arterial pressure	Mean arterial pressure	Mean arterial pressure
Systolic blood pressure	Systolic blood pressure	Systolic blood pressure	Systolic blood pressure
Diastolic blood pressure	Diastolic blood pressure	Diastolic blood pressure	Diastolic blood pressure
Heart rate	Heart rate	Heart rate	Heart rate

Table 3.4 Respiratory Parameters			
1-4 hours prior to transfusion	During transfusion	1-4 hours post transfusion	5-10 hours post transfusion
Mean airway pressure FiO_2 Respiratory Rate % time out of target band	Mean airway pressure FiO_2 Respiratory Rate % time out of target band	Mean airway pressure FiO_2 Respiratory Rate % time out of target band	Mean airway pressure Respiratory Rate

Table 3.5 Definitions of neonatal transfusion associated morbidities		
Acute Respiratory Distress	Tachycardia	Increased mean blood pressure
Sustained rise in FiO_2 >20%	Sustained rise in heart rate ≥20% up to 4 hours post transfusion	Sustained rise in MBP ≥20% up to 4 hours post transfusion
Sustained rise in respiratory rate >15 per minute	Any rise up to 10 hours post exposure	Any rise up to 10 hours post exposure

In addition, a number of additional measures of cardio-respiratory function were derived from the physiological data including cardiac output (CO), pulse pressure (PP), oxygen delivery index and respiratory severity score (RSS). In the current study, to ensure minimal discomfort and interruption to normal clinical care, a non-invasive technique was used to estimate cardiac output. Validated in adult and paediatric populations against invasive measures of cardiac output [237], cardiac output was defined as:

$$\mathrm{CO} = \frac{\text{Pulse Pressure}}{(\text{Systolic Blood Pressure} + \text{Diastolic Blood Pressure}) \times \text{Heart Rate}}$$

With pulse pressure (PP) defined as systolic blood pressure minus diastolic blood pressure.

The RSS is a non-invasive surrogate for oxygenation index and has been validated in mechanically ventilated preterm infants [238]. RSS was calculated from:

$$\mathrm{RSS} = \text{Mean Airway Pressure} \times \text{Fraction of Inspired Oxygen}$$

3.3.3 Sex of Transfusion Donor

To determine the sex of each transfusion donor, pack and donor number were recorded allowing re-identification of donor sex by The Australian Red Cross Blood Service.

3.3.4 Outcome Assessments

While the primary outcome of this study was the immunological changes following the transfusion a number of neonatal morbidity measures were collected and defined as the following:

- BPD: based on the need for ventilatory support or supplemental oxygen at 36 weeks postmenstrual age.
- IVH: for the purposes of this study, grade III and IV IVH were included as an outcome. This grading system is based on standard criteria developed by Papile [239]. The study centres followed a ultrasound protocol dictating a minimum number of scans (day 1-2, day 7 and day 42).
- ROP: a grading of 3 or greater was considered as an outcome, as per the current standard international criteria [240].
- NEC: a grading of stage 2 or greater was used based on the criteria proposed by Bell [241].
- Nosocomial infection: blood culture positive sepsis at any point during primary hospital admission.

3.4 Measurement of Neonatal Immune Function

3.4.1 High Sensitivity T-Cell Multiplex Assay

Circulating cytokine levels for each transfusion sample were analysed using a Milliplex® 96 well Human High Sensitivity T-Cell Magnetic Bead panel multiplex ELISA. Detection limits and inter-assay and intra-assay precision are listed below as per manufacturer. (Tables 3.6 and 3.7).

Table 3.6 Minimal detectable cytokine concentrations for Human High Sensitivity T-Cell Magnetic Bead Panel

Analyte	Overnight Protocol
	Minimum Detectable concentration (pg/mL)
IFNγ	0.48
IL-10	0.56
IL-12 (p70)	0.15
IL-17A	0.33
IL-1β	0.14
IL-6	0.11
IL-8	0.13
TNF	0.16

Table 3.7 Inter-assay and Intra-assay precision for Human High Sensitivity T-Cell Magnetic Bead panel

Analyte	Overnight Protocol	
	Intra-assay %CV	Inter-assay %CV
IFNγ	<5%	<20%
IL-10	<5%	<20%
IL-12 (p70)	<5%	<15%
IL-17A	<5%	<20%
IL-1β	<5%	<15%
IL-6	<5%	<20%
IL-8	<5%	<15%
TNF	<5%	<15%

All reagents, controls and standards were prepared according to manufacturer instructions. The multiplex ELISA was run on a MAGPIX® multiplexing system with xPONENT® software. There was no cross-reactivity between antibodies or analytes on this panel.

3.4.2 Neonatal Sepsis Assay

Markers for endothelial activation were measured for each transfusion sample, analysed using a Milliplex® 96 well human sepsis bead panel multiplex enzyme-linked immunosorbent assay (ELISA). The detection limits and inter-assay and intra-assay precision are listed below as per manufacturer (Tables 3.8 and 3.9).

Table 3.8 Minimal detectable cytokine concentrations for Human Sepsis Bead panel

Analyte	Overnight Protocol
	Minimum Detectable concentration (pg/mL)
MIF	2.7
sICAM-	17.7
sFasL	3.7
sFas	4.4
sVCAM-1	10.7
PAI-1	3.2

Table 3.9 Inter-assay and Intra-assay precision for Human Sepsis Bead panel

Analyte	Overnight Protocol	
	Intra-assay %CV	Inter-assay %CV
MIF	1.6	13.6
sICAM-	0.8	11.5
sFasL	0.7	11.6
sFas	1.2	9.7
sVCAM-1	0.8	10.6
PAI-1	1.2	9.9

As for the Human High Sensitivity T-Cell Magnetic Bead panel multiplex ELISA, all reagents, controls and standards were prepared according to manufacturer instructions and each plate was run on the MAGPIX® multiplexing system with xPONENT® software. There was no cross-reactivity between antibodies or analytes on this panel.

3.5 Statistical Analysis

3.5.1 Sample Size Calculation

A range of pro-inflammatory cytokines have previously been demonstrated to increase following exposure to leukodepleted unwashed PRBCs. However, there is inconsistency in the timing of post-transfusion measurement in the literature. IL-17A has been shown to be increased at multiple time-points post-transfusion [37] therefore the current studies sample size was based on the Δchange (the difference between post- and pre-concentrations) in IL-17A

from pilot data in 40 extremely preterm infants exposed to unwashed PRBCs. The mean Δchange was 15.3 pg/ml with a standard deviation of 13.1 pg/ml. It was calculated that a sample size of 77 infant per group would provide 90% power to detect a group difference of 6.6 pg/ml (on-half of a SD) of the mean IL-17A with an α =0.05. Since it could not be determined prospectively which infants would be transfused, enrolment exceeded that necessary for the sample size calculation to ensure adequate power for the primary aim in transfused subjects.

3.5.2 Cytokine and Endothelial Marker Analysis

Extremely preterm infants will have a median number of 3-5 transfusion exposures during their primary hospital admission. Therefore, we made an a priori decision to determine transfusion associated changes in circulating cytokines and markers of endothelial activation for the first 4 transfusions. Analysis was conducted blinded to study allocation. All descriptive statistics are presented as median (interquartile range) or number (percentage) where appropriate.

As the plasma cytokine concentrations were not normally distributed the Friedman test was used to assess differences between baseline cytokine concentrations prior to each transfusion exposure with the alpha level set at 0.01 to adjust for multiple comparisons. Pre- and post-transfusion levels of plasma cytokines are presented as median (interquartile range) with differences assessed using the Wilcoxon signed rank test with the p value <0.05 considered significant. Within subject temporal changes, in the pre- to post-transfusion Δchanges in plasma cytokines and markers of endothelial activation, in each group across the three transfusion exposures were analysed using a Linear Mixed Model. Heterogenous compound symmetry was used as the repeated covariance type and LSD as the confidence interval adjustment, to compare changes within treatment groups. To investigate whether transfusion related changes in cytokines and markers of endothelial activation were influenced by gestational age, sex, age at first transfusion, and pre-transfusion haemoglobin, these variables were included as co-variates. Data were analysed using the Statistical Package for the Social Sciences (SPSS v26; IBM SPSS, Chicago, IL).

3.5.2 Physiological Response to Transfusion Analysis

Cardiovascular and respiratory parameters from preterm infants <29 weeks' gestation prior, during and post their first transfusion exposure were collected. Demographic data were compared using ANOVA or Mann-Whitney U depending on distribution and differences in frequencies for categorical variables tested using the χ^2 test or Fisher's exact test. To account for missing data points temporal changes in cardio-respiratory variables were analysed using Linear Mixed Models with transfusion pack type (unwashed or washed) used as a fixed factor and post-hoc paired t-tests with a Bonferroni correction. The delta (post-pre-transfusion difference between the 4-hour post transfusion time point and the immediate pre-transfusion time point) was calculated for both cardio-respiratory parameters and inflammatory cytokines and the relationship between the measures of interest assessed using spearman's rho. Data were analysed using the Statistical Package for the Social Sciences (SPSS v26; IBM SPSS, Chicago, IL).

3.5.3 Sex of the Donor Analysis

Study groups were defined by the donor sex (those who received PRBCs from only male donors for the first three transfusions or those who received any PRBCs from a female donor) and whether they received unwashed or washed PRBCs. Cytokines and markers of endothelial activation data were therefore analysed as 4 groups: unwashed PRBCs male only donors; unwashed PRBCs any female donor; washed PRBCs male only donors; and washed PRBCs any female donor.

Demographic data is presented as median (IQR) or N (%). Fisher's Exact Test or Pearson Chi-Square used for comparison between groups. As the cytokine and endothelial markers were not normally distributed the Kruskal-Wallis test was used to compare differences between pre- and post-transfusion concentrations for the 4 groups with a $p<0.05$ considered statistically significant. Where the pre- post-transfusion change was significant for more than one of the

groups, the magnitude of the difference (Δchange) was compared by the Kruskal-Wallis test with Dunn's post hoc test to investigate differences between the individual groups, with a $p<0.01$ used to correct for multiple comparisons. Further, hierarchical multiple regression was used to assess the impact of exposure of the four transfusion exposure groups (unwashed PRBCs male only donors; unwashed PRBCs any female donor; washed PRBCs male only donors; and washed PRBCs any female donor) on the magnitude of the change, after controlling for gestational age, newborn sex, and age at first transfusion. Data were analysed using the Statistical Package for the Social Sciences (SPSS v26; IBM SPSS, Chicago, IL).

Chapter 4

Baseline Characteristics at Study Entry and Long-term Outcomes

4.1 Clinical Characteristics of Enrolled Infants not Transfused versus Randomised and Transfused

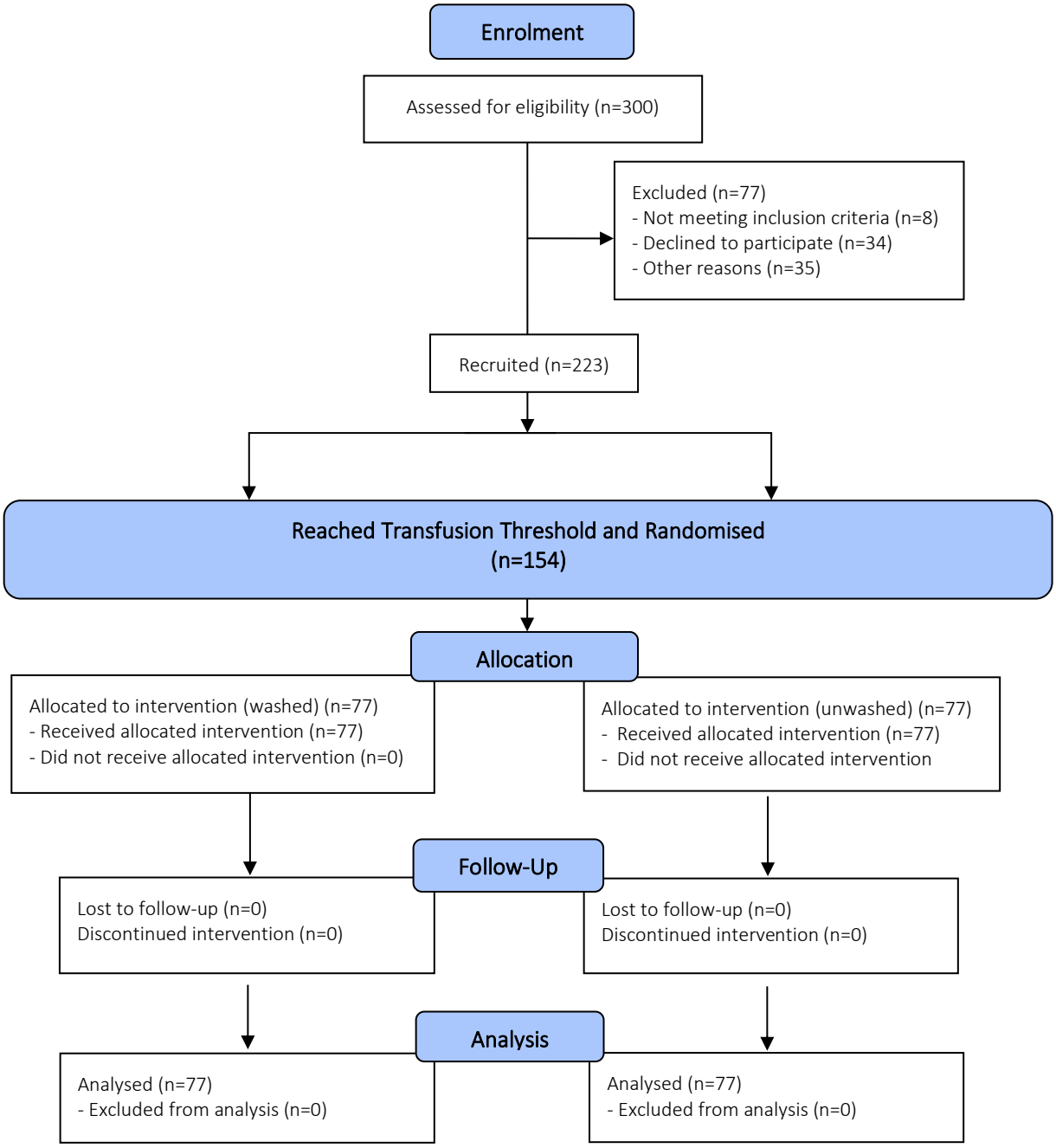

Figure 4.1 Study consort diagram

During the study period, 223 infants were enrolled with 154 reaching the PINT haemoglobin trigger resulting in randomisation and transfusion (Figure 4.1). The clinical characteristics of those enrolled but not transfused versus those randomised are shown in Table 4.1. Maternal antenatal diagnoses were collected from the South Australian Perinatal data set, with diagnostic criteria as per South Australian Perinatal Practice Guidelines [242]. Those that required

a transfusion were a significantly younger gestation compared to those who were not transfused (p<0.01).

Table 4.1 Antenatal and Neonatal Clinical Characteristics

	Enrolled (n= 223)	Transfused (n= 154)	P
Gestation	28 (25 – 28^{+6})	26 (23 – 28^{+6})	<0.01
Male	35 (61)	97 (63)	0.47
Birth Weight, grams	1060 (446 – 1625)	800 (380 – 1690)	0.1
IUGR (<10^{th} percentile)	4 (7)	23 (15)	0.08
Multiple Birth	17 (30)	39 (26)	0.33
Chorioamnionitis, histological	8 (14)	52 (35)	<0.01
Antenatal Steroids			
None	2 (4)	11 (7)	
Incomplete	2 (4)	27 (18)	<0.05
Complete	42 (79)	94 (64)	
Repeat	7 (13)	16 (11)	
SIP	0 (0)	6 (4)	0.13
NEC	2 (4)	7 (5)	0.52
PVL	0 (0)	5 (3)	0.19
IVH	3 (5)	20 (13)	0.07
ROP	0 (0)	37 (25)	<0.001
BPD	16 (28)	86 (59)	<0.01
Length of ventilation, days	1 (0 – 9)	10 (0 – 55)	<0.05
Length of stay, days	59 (50 – 71)	95 (78 – 111)	<0.05
Sepsis, culture positive	4 (7)	13 (9)	<0.05
Death	2 (4)	13 (9)	0.16

Data presented at median (IQR) or N (%). Kruskal Wallis test and Fisher's Exact test or Pearson Chi-Square used for comparison between groups.

However, there was no significant difference in birthweight. Transfused infants were more likely to be born in the context of histological chorioamnionitis (p<0.01) and be exposed to a complete course of antenatal steroids (p<0.05), defined as Betamethasone 24mg divided dose completed between 12 and 36 hours (two intramuscular 11.4mg doses 24 hours apart) or Dexamethasone 24mg divided dose completed between 24 and 40 hours (four intramuscular doses of 6mg 12 hours apart) [243]. Finally, transfused infants had significantly greater rates of ROP (p<0.001), BPD (p<0.01), days of mechanical ventilation (p<0.05), length of stay (p<0.05) and culture positive sepsis (p<0.05). No other differences were seen between enrolled and randomised and transfused infants.

4.2 Clinical Characteristics of Transfused Newborns by Neonatal Unit

Infants were recruited in two nurseries, The Women's and Children's Hospital (WCH) and the Flinders Medical Centre (FMC). The Women's and Children's site is the sole maternal-fetal medicine service in South Australia. Therefore, the patient populations cared for by the nurseries differed, with all high-risk pregnancies (including surgical and cardiac diagnoses which were excluded from enrolment) delivered at WCH. The transfusion threshold did not differ between the two sites with the PINT haemoglobin trigger implemented at both. However, transfusion protocols within the two units were not identical. While infants born at the Women's and Children's hospital were fasted prior to, throughout the PRBC transfusion, and for 4 hours after, infants at the FMC nursery were not routinely fasted. The clinical characteristics of the randomised infants at the individual sites are shown in Table 4.2. More male infants were randomised at the FMC site (p=0.01). In addition, total length of stay was longer at the FMC site compared to WCH. While this may reflect individual practice differences between the two nurseries, it is also impacted on by the geographical nature of hospital down transfer in South Australia with more limited capacity to care for convalescent preterm newborns in the level 2, 3 and 4 nurseries aligned with FMC out with metropolitan Adelaide compared to those associated with WCH. No other significant differences were seen between the two nurseries.

4.3 Clinical Characteristics by Red Blood Cell Type Exposure

Of the 154 infants transfused during the study period, 77 were allocated to receive washed PRBCs and 77 were allocated to unwashed PRBCs. Maternal and neonatal baseline characteristics are shown in Table 4.3. No significant differences were seen between the two groups.

4.4 Baseline Transfusion Characteristics by Pack Type

There were no differences in transfusion characteristics between the two study arms (Table 4.4.) Infants in both groups were exposed to their first transfusion within the first week of postnatal life, 4 days (2-11) versus 3 days (2-7) (unwashed versus washed). For those infants who required additional PRBC transfusions, the median age at the second transfusion was 6 versus 7 days, the third, 13 versus 19 days, and the fourth, 21 versus 25 days. Despite a transfusion protocol being used for the current trial, a number of infants were transfused out of the haemoglobin trigger. All received the allocated transfusion product in line with original allocation. In almost all cases this was a transfusion requested when a haemoglobin was recorded higher than what was required to trigger a transfusion. This was a result of clinical instability and was at the discretion of the on-call clinical team. However, there were no differences noted between the two groups in relation to transfusions occurring out of the trial protocol.

Table 4.2 Antenatal and Neonatal Clinical characteristics

	WCH (n=128)	FMC (n=29)	P
Gestation	26 (24 – 27)	25 (25 – 27)	0.1
Male	73 (58)	24 (83)	0.01
Birth Weight, grams	830 (650 – 966)	800 (720 – 1020)	0.49
IUGR	21 (17)	3 (10)	0.57
Multiple Birth	35 (28)	4 (14)	0.15
Chorioamnionitis, histological	44 (35)	9 (33)	1.0
Antenatal Steroids			
None	10 (8)	1 (4)	
Incomplete	23 (19)	5 (18)	0.52
Complete	75 (61)	21 (75)	
Repeat	15 (12)	1 (4)	
SIP	4 (3)	3 (10)	0.12
NEC	7 (6)	0 (0)	0.35
PVL	4 (3)	2 (7)	0.32
IVH	18 (14)	2 (7)	0.37
ROP	30 (24)	9 (31)	0.48
BPD	71 (57)	19 (68)	0.4
Length of ventilation, days	11 (3 – 19)	6 (2 – 23)	0.8
Length of stay, days	88 (71 – 108)	100 (86 – 123)	0.03
Sepsis, culture positive	26 (21)	6 (21)	1.0
Death	11 (9)	2 (7)	1.0

Data presented at median (IQR) or N (%). Kruskal Wallis test and Fisher's Exact test or Pearson Chi-Square used for comparison between groups.

Table 4.3 Baseline Antenatal and Neonatal Characteristics

	Unwashed PRBCs (n=77)	Washed PRBCs (n=77)	P
Maternal			
Age, years	30 (26 – 35)	32 (27 – 36)	0.18
Parity	1 (0 – 1)	1 (0 – 2)	0.24
Mode of Delivery			
SVD	29 (38)	26 (34)	0.74
LSCS	48 (62)	51 (66)	
Steroids			
None	6 (8)	5 (7)	
Incomplete	13 (17)	15 (20)	0.8
Complete	48 (63)	49 (64)	
Repeat	9 (12)	7 (9)	
Antibiotics	31 (40)	31 (40)	1.0
Magnesium Sulphate	48 (62)	52 (67)	0.15
Chorioamnionitis, histological	30 (39)	23 (30)	0.3
High Vaginal Swab	20 (26)	19 (24)	1.0
Diabetes, any	3 (4)	6 (8)	0.5
Premature rupture of membranes	22 (29)	28 (36)	0.3
Antepartum Haemorrhage	26 (34)	20 (26)	0.38
Preeclampsia	2 (3)	5 (6)	0.27
Multiple Birth	22 (29)	17 (22)	0.46
Neonatal			
Gestation, weeks	26 (24 – 27)	26 (25 – 27)	0.72
Male	50 (65)	47 (61)	0.74
Birth Weight, grams	800 (660 – 966)	830 (650 – 990)	0.8
IUGR	9 (12)	15 (19)	0.27
Intubated at Delivery	31 (40)	40 (52)	0.2
APGAR 5 minutes	7 (6 – 8)	7 (6 – 8)	0.9

Data presented at median (IQR) or N (%). Kruskal Wallis test and Fisher's Exact test or Pearson Chi-Square used for comparison between groups.

Table 4.4 Baseline Transfusion Characteristics

Transfusion Exposure	1			2			3			4		
	UW	W	p	UW	W	p	UW	W	p	UW	w	p
Postnatal Age (days)	4 (2 – 11)	3 (2 – 7)	0.64	6 (3 – 11)	7 (3- 18)	0.88	13 (6 – 26)	19 (7 – 28)	0.58	21 (10 – 32)	25 (11 – 35)	0.52
Pre-transfusion Hb (g/l)	122 (103 - 135)	122 (102-131)	0.54	115 (97 -126)	111 (95 - 123)	0.49	105 (89 -126)	100 (84 -115)	0.16	98 (84 - 109)	96 (82 -113)	1.0
Post-transfusion Hb (g/l)	133 (122-149)	135 (124-146)	0.89	133 (119-144)	131 (115-144)	0.75	126 (118-141)	127 (114 -137)	0.82	123 (112-132)	121 (111-134)	0.86
Transfusion out of protocol	22 (28)	17 (22)	0.7	15 (25)	8 (13)	0.4	13 (28)	11 (23)	0.84	10 (33)	8 (21)	0.88

Data presented at median (IQR) or N (%). UW = Unwashed PRBC group W= washed PRBC group. Kruskal Wallis Test and Pearson Chi-Square used for comparison between groups.

4.4 Long-term Outcomes by Red Blood Cell Type Exposure

There were no significant differences seen between the two groups in relation to long-term outcomes clinical outcomes. Of the 141 infants that reached hospital discharge, their clinical outcomes are shown in Table 4.5.

Table 4.5 Long-term Outcomes

	Unwashed PRBCs	Washed PRBCs	P
RDS	74 (96)	72 (94)	0.72
Surfactant	64 (83)	70 (91)	0.23
Sepsis, culture positive	20 (26)	12 (16)	0.5
Early (<48 hours)	1	3	
Late	19	9	
Total Transfusions	3 (2 – 6)	3 (2 – 6)	1.0
Emergency Transfusion	21 (27)	21 (27)	1.0
Received Platelets or Plasma	23 (31)	21 (29)	0.9
SIP	5 (6)	2 (3)	0.44
NEC	5 (6)	2 (3)	0.44
PVL	2 (3)	4 (5)	0.7
IVH	9 (12)	11 (14)	0.8
ROP	21 (27)	18 (23)	0.7
BPD	45 (58)	45 (58)	1.0
Length of ventilation, days	10 (2 – 22)	11 (4 – 18)	0.9
Postnatal Steroids	38 (49)	40 (52)	0.87
Length of stay, days	91 (78 – 110)	92 (68 – 111)	0.49
Death	5 (6)	8 (10)	0.4

Data presented at median (IQR) or N (%). Kruskal Wallis test and Fisher's Exact Test or Pearson Chi-Square used for comparison between groups.

While there was no difference between the median number of transfusions infants in the unwashed and washed groups received during their hospital admission, the range was large. Out of the 154 randomised infants, 33 (21%) had a single transfusion, 24 (16%) had two, 22 (14%) had three and 19 (12%) had four in total. The remaining 37% of the randomized infants had five or more PRBC transfusions prior to discharge with the maximal exposure being eighteen PRBC transfusion events.

4.5 Summary

From the above data, it is clear that the study participants represent a widely generalisable population of extremely preterm newborns cared for in a tertiary neonatal intensive care setting. Importantly, other than a higher number of male infants recruited and randomised at the FMC site the populations of infants at both were similar. It is unsurprising that those infants who ultimately required one of more transfusions were of lower gestational age, as is the increased incidence significant morbidity associated with extremely preterm birth in this group, including length of ventilation, length of stay, BPD, ROP and sepsis, though similar rates of death. The higher rates of morbidity are also likely to be related to the higher incidence of chorioamnionitis and intra-uterine growth restriction and lower rates of completed antenatal maternal steroid administration, pregnancy complications known to result in greater instability in the neonatal period.

Critically, no significant differences in any baseline antenatal or neonatal clinical characteristics were observed between the unwashed and washed groups. Further, the baseline transfusion characteristics, in particular postnatal age at transfusions 1 to 4 and degree of pre-transfusion anemia, were also identical between the groups. Taken together, this strengthens the likelihood that any significant difference in the transfusion recipients inflammatory and physiological post-transfusion responses between the unwashed and washed PRBC groups is not confounded by other clinical variables and is due to the difference in blood product exposure. Although no differences were seen in long term outcomes, it is important to keep in mind, this study was not powered to assess long term outcomes and as a result does not accurately reflect this.

Chapter 5

Physiological Response to Red Blood Cell Exposure

5.1 Introduction

Iatrogenic blood loss, an immature bone marrow response to falling haemoglobin and shortened red blood cell life span all contribute to preterm infants being the highest transfused population. In fact, it has been estimated that in a single week, a preterm infant may lose between 15 to 30% of their total blood volume [35]. Therefore, almost all extremely preterm or low birth weight infants will require a transfusion during their primary hospital admission [237].

Theoretically, transfusion should be beneficial with improvements in oxygen-carrying capacity resulting in alterations in cardiac output and consumption in addition to an overall increase in physiological stability [238]. However, in adult patients, transfusions may result in acute adverse physiological responses referred to as TACO and TRALI [244]. While there are accepted definitions for both these conditions in adults there is still debate as to their existence in the preterm infant [245]. While the preterm infant is at risk of adverse outcomes due to fluctuations in cardio-respiratory stability due to anaemia, it remains unclear if this population also exhibit adverse consequences intrinsic to the transfusion exposure itself [245]. In particular, the lack of an agreed classification for TACO or TRALI in this population has resulted in these important consequences of transfusion exposure being seldom recognised and potentially under-reported.

A range of morbidities related to preterm birth have repeatedly been shown to be independently associated with volume and number of transfusion exposures [36]. Previous studies have demonstrated that in the 'stable' late preterm infant (>29 weeks' gestation) a transfusion corrects the physiological response to anaemia and reduced oxygen-carrying capacity with a decrease in cardiac output, heart rate and lactate [120]. However, the majority of transfusion exposure is predominantly in the very early neonatal period in the most preterm infants. This is a period of significant physiological instability characterised by respiratory

disease such as hyaline membrane disease and cardiovascular dysfunction manifesting as poor cardiac function and hypotension. It is also likely that the pathophysiologic processes that ultimately result in significant neonatal morbidity begin during this period. This raises the possibility that any additional cardio-respiratory dysfunction, potentially as a result of adverse responses to transfusion, could contribute to an increased risk of morbidity and mortality.

Multiple factors contribute to the development of significant neonatal morbidities such as BPD, IVH and NEC, including transfusion exposure. Much attention has been given to the contribution of transfusion related immunomodulation as a potential link between transfusion and adverse outcome [246]. However, it remains unknown if the potential harmful consequences of transfusion are due to acute adverse physiological responses, analogous with TACO and TRALI in other critically ill patient populations, inflammatory processes related to TRIM or both. To answer this complex question requires detailed characterisation of the cardio-respiratory responses to transfusion in the preterm infant particularly focusing on any physiological markers of deterioration in cardio-respiratory function which could be consistent with those observed in other patient groups in whom TACO and/or TRALI is a recognised complication. The aim for this chapter was to determine the cardio-respiratory response to transfusion exposure and ascertain if these responses differed with transfusion with washed versus unwashed leukodepleted PRBCs. Further, I investigated whether the cardio-respiratory responses to transfusion demonstrated any relationship with transfusion-related alterations in pro-inflammatory cytokines to explore possible links between TRIM and physiological response to transfusion exposure.

5.2 Methods

5.2.1 Cardio-respiratory Data Collection

Cardiovascular and respiratory parameters from preterm infants <29 weeks' gestation prior, during and post their first transfusion exposure were collected. Physiological parameters

recorded included heart rate, systolic and diastolic blood pressure, mean arterial pressure, mean airway pressure, fraction of inspired oxygen, and respiratory rate. As described in Chapter 3, physiological data was collected from immediately prior to the transfusion, throughout and then up to 10 hours following their transfusion exposure retrospectively from the subject's medical record. SBP, DBP and MBP was measured invasively from an umbilical arterial catheter or peripheral arterial catheter, when present, or by non-invasive blood pressure measurement. Heart rate and respiratory rate were measured using ECG electrodes and the impedance pneumography waveform from the patients electronic monitoring (Phillips 600X monitoring system). For ventilated infants, mean airway pressure and fractional inspired oxygen was derived from the ventilator (Drager Infiniti V500). For those infants on non-invasive respiratory support (BCPAP or Optiflow) the set end-expiratory pressure and inspired oxygen was recorded.

In addition, CO, RSS and systemic vascular resistance (SVR) were calculated from these physiological variables, in addition to percentage time out with saturation target band, defined as 90 – 94% which was calculated from an hourly histogram using in-bedded software on the Phillips 850 monitors. Due to ethical restrictions on additional invasive investigations in this population of extremely preterm infants, cardiac output was defined as:

$$\mathrm{CO} = \frac{\text{Pulse Pressure}}{(\text{Systolic Blood Pressure} + \text{Diastolic Blood Pressure}) \times \text{Heart Rate}}$$

This is a well-validated measure of cardiac output used in the adult population and was adapted for the current population [237]. RSS is a proxy measure of oxygenation indices that has been validated in the preterm population [247]. RSS was calculated from mean airway pressure and fraction of inspired oxygen.

$$\mathrm{RSS} = \text{Mean Airway Pressure} \times \text{Fraction of Inspired Oxygen}$$

Percentage of time out of target band was calculated hourly and generated using the Nellcor oximetry algorithm in combination with the Philips Intellivue Mx 800 monitor. Oximetry data was obtained using a 15 second sampling time and presented hourly allowing the generation of a value of percentage of time out of target band. The saturation target band was based on the Cochrane individual patient meta-analysis for oxygen saturation targets [248].

In the normal clinical setting, SVR is calculated using cardiac output, mean arterial pressure and central venous pressure. In the current study, SVR was calculated in the simplified form using mean arterial pressure and cardiac output.

$$\text{SVR} = \text{Mean Arterial Pressure} \div \text{Cardiac Output}$$

5.2.2 Plasma Sample Collection and Cytokine Analysis

Plasma samples were collected from infants immediately prior to transfusion exposure and 4-6 hours post transfusion completion. Samples were processed as previously described in section 3.3.1 Plasma Collection. Cytokines of interest including IFNγ, IL-10, IL-12, IL-17A, IL-1β, IL-6, IL-8 and TNF were assessed using Multiplex ELISA. These measures were paired with baseline and 4-hour post cardio-respiratory measurements.

5.2.3 Statistical Analysis

Clinical characteristics are presented as median (interquartile range) or number (percentage) where appropriate. Demographic data were compared using ANOVA or Mann-Whitney U depending on distribution and differences in frequencies for categorical variables tested using the χ^2 test or Fisher's Exact test. To account for missing data points, temporal changes in cardio-respiratory variables were analysed using Linear Mixed Models with transfusion pack type (unwashed or washed) used as a fixed factor and post-hoc paired t-tests with a Bonferroni correction. The delta (post-pre-transfusion difference between the 4-hour post transfusion time point and the immediate pre-transfusion time point) was calculated for both cardio-respiratory parameters and inflammatory cytokines and the relationship between the

measures of interest assessed using spearman's rho. Data were analysed using the Statistical Package for the Social Sciences (SPSS v26; IBM SPSS, Chicago, IL).

5.3 Results

One hundred and fifty-four extremely preterm infants had physiological data recorded prior, during and following their first transfusion exposure (one-hour pre transfusion; throughout the 3-hour PRBC infusion and four hours post). Neonatal transfusion characteristics are presented in Table 5.1.

Table 5.1 Transfusion Characteristics at First Transfusion

	Unwashed PRBCs	Washed PRBCs	P
Postnatal Age, days	4 (2 – 11)	3 (2 – 7)	ns
Pre-transfusion Hb	122 (103 - 135)	122 (102 – 131)	ns
Post-transfusion Hb	133 (122-149)	135 (124 – 146)	ns
Transfusion out of protocol	22 (28)	17 (22)	ns
Respiratory Support			
Non-invasive	32 (42)	24 (31)	ns
Invasive	44 (57)	52 (68)	
Inotropes	26 (34)	29 (38)	ns
PDA	33 (43)	32 (42)	ns

Data presented at median (IQR) or N (%). Fisher's Exact Test or Pearson Chi-Square used for comparison between groups. Echocardiographically significant PDA was defined as per Kluckow,M. et al, 1995 [249]

Both the unwashed and washed groups received their first transfusion within the first week of postnatal life. No statistically significant differences were seen between the groups in regard to any of the transfusion characteristics. At the time of first transfusion exposure, the majority of infants were receiving invasive ventilatory support (57% in the unwashed group and 68% in the washed group). In both groups, almost half had a clinically significant PDA and approximately 30% were receiving inotropic support.

Haemoglobin, packed cell volume and lactate levels were collected immediately prior to and following the transfusion. Pre-transfusion, there was no difference between the unwashed and washed groups in relation to any of these measures. Following the transfusion, there were significant increases in haemoglobin ($p<0.001$) and packed cell volume ($p=0.003$) for both unwashed and washed groups, with the magnitude of the increase similar between the groups. pH decreased following exposure to unwashed blood, although the fall did not reach significance ($p=0.059$), with no change in pCO_2. For infants exposed to washed blood, there were no changes in pH or pCO_2. No change in lactate was seen following transfusion for either group ($p=0.754$) (see Figure 5.1)

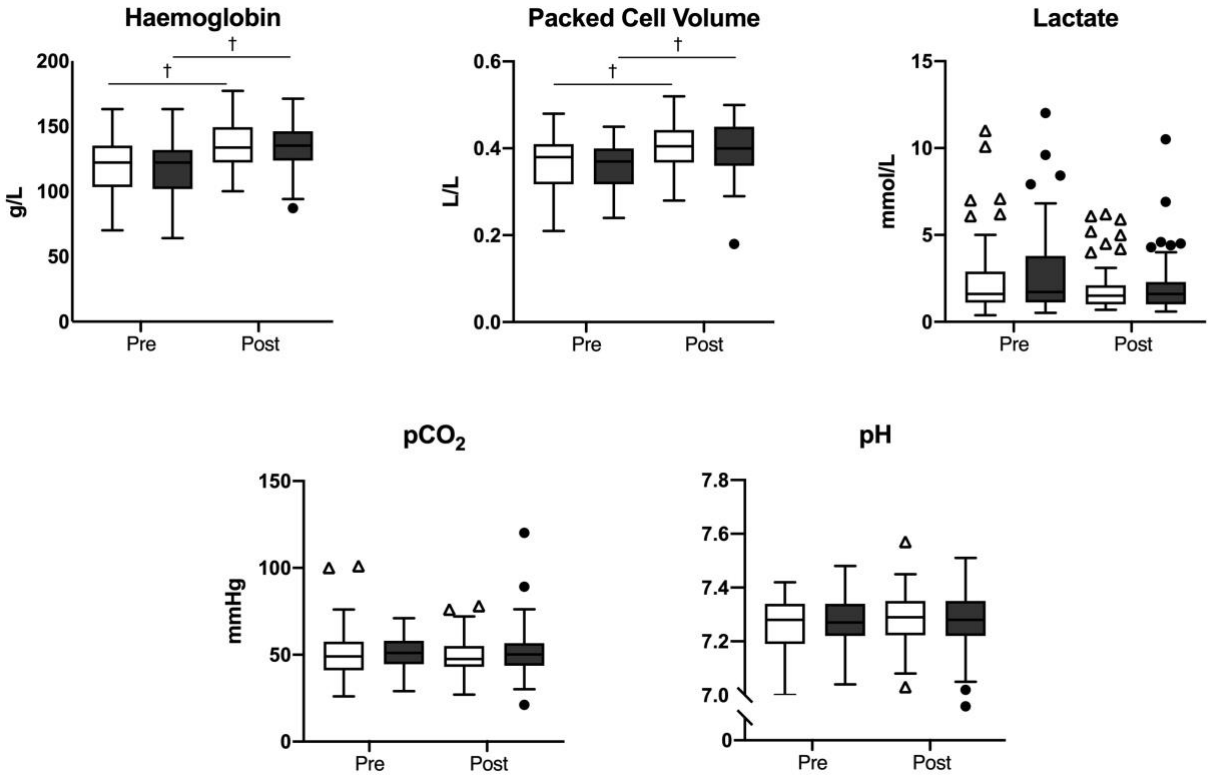

Figure 5.1 Haematological response to first transfusion. Unwashed shown in the open box and whisker, washed shown in the grey box and whisker. † $p<0.001$.

Temporal changes in cardiovascular parameters in relation to transfusion with unwashed or washed PRBCs are shown in Figure 5.2. Overall, cardiac output fell over time (t=-2.75 p<0.01 95% CI -0.9 to -1.4). When assessing the effect of pack type, infants exposed to washed PRBCs exhibited a greater decrease in CO (t= -2.57, p<0.05, 95% CI -0.62 to -4.68). with a -0.50 fall at each time point (p<0.01). SVR increased with time (t=4.24, p<0.001, 95% CI 0.02 to 0.05) and was influenced by transfusion type (t=2.19, p=0.03, 95% CI 0.01 to 0.16), being higher in infants transfused with washed PRBCs.

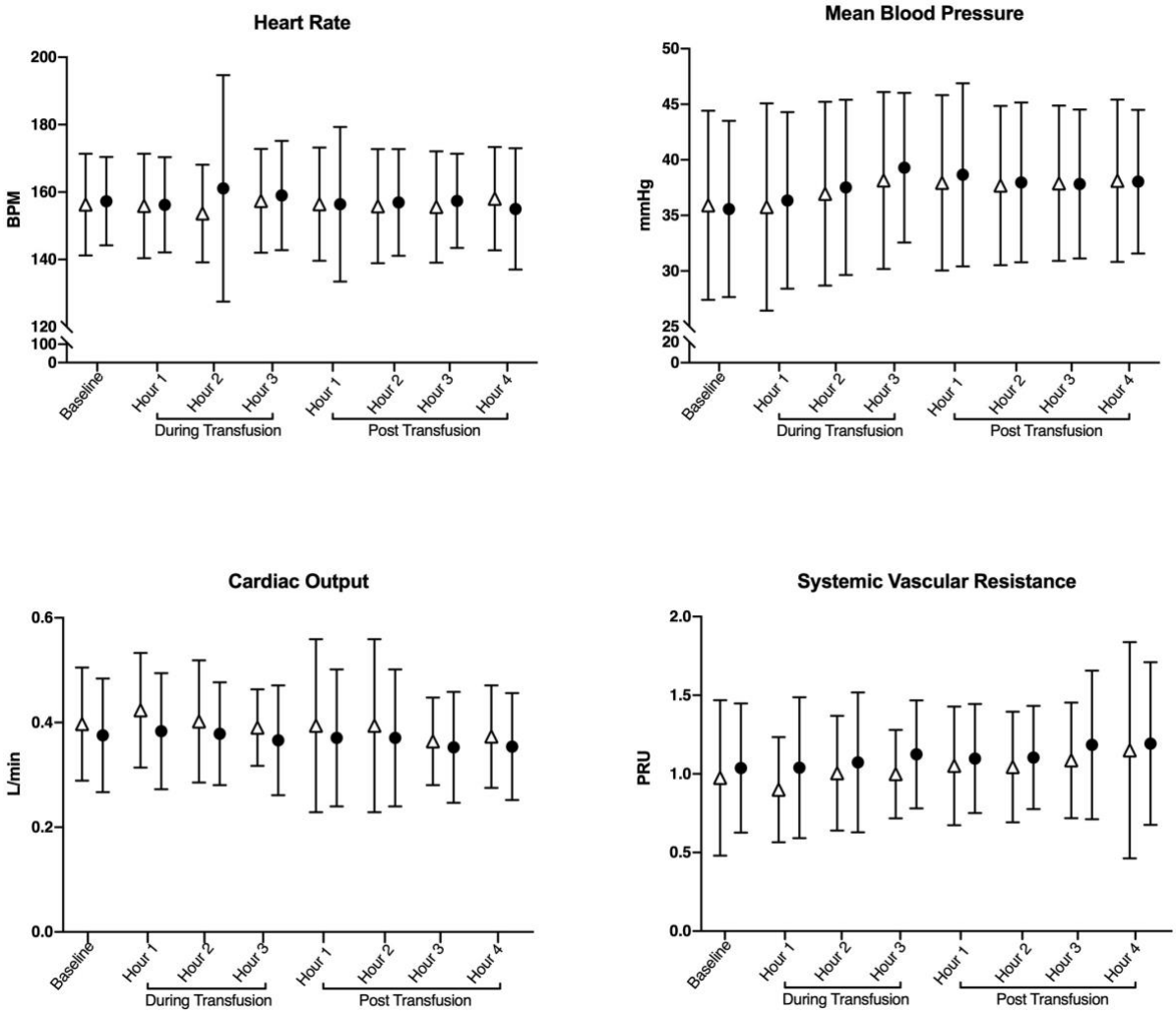

Figure 5.2 Cardiovascular changes following first transfusion exposure. Δ Unwashed
- Washed

While an increase in DBP (t=4.23, p>0.001 95% CI 0.28 to 0.78) occurred over time, no effect of transfusion type was seen. No temporal or transfusion type specific changes were observed

in HR, SBP, MBP, or PP. Temporal changes in respiratory parameters in relation to transfusion with unwashed or washed PRBCs are shown in Figure 5.3.

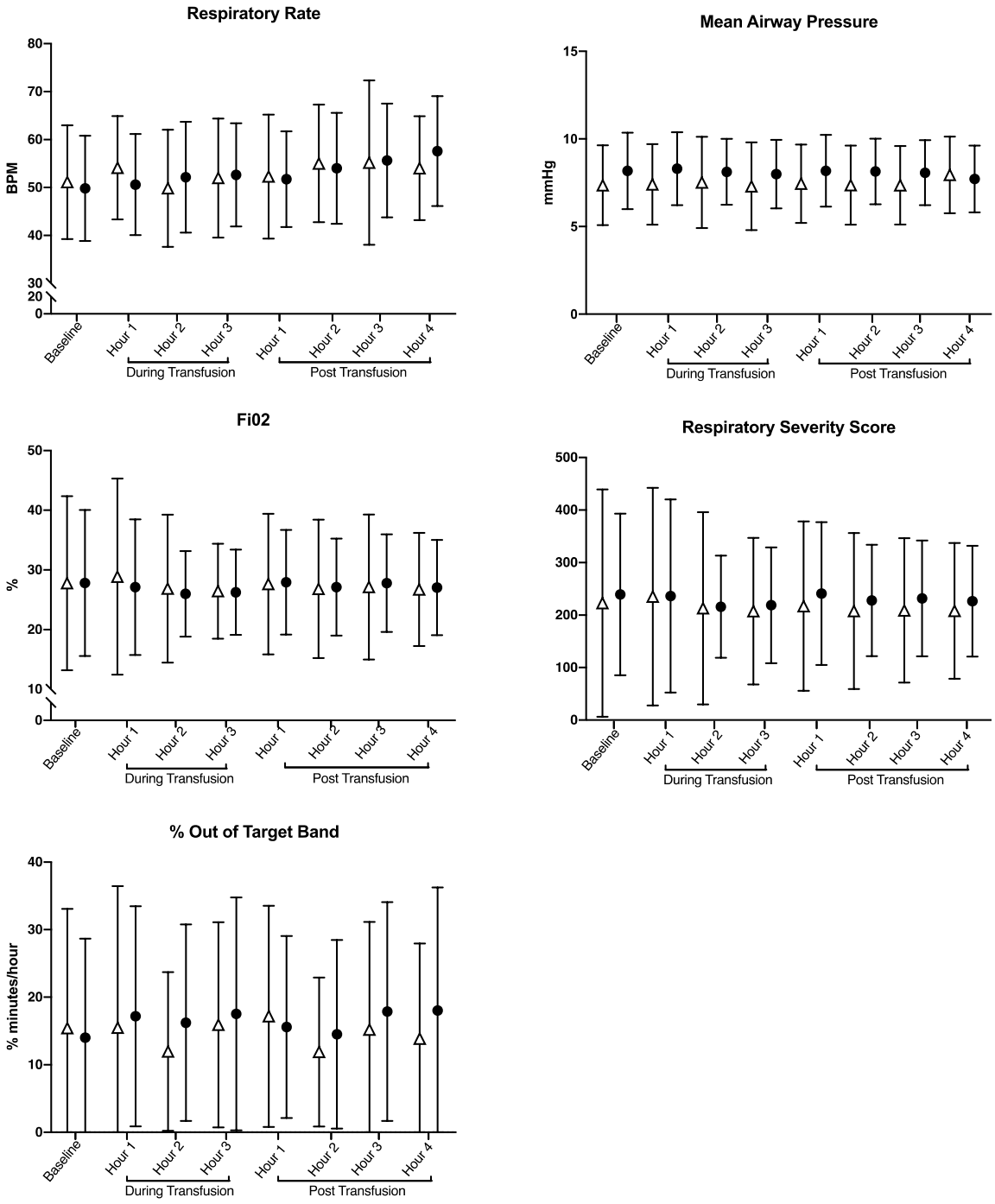

Figure 5.3 Respiratory changes following first transfusion exposure. △ Unwashed ● Washed

RR increased with time (t=1.96, p<0.05, 95% CI 0.2 to 1.7) but was not influenced by pack type. MAP did not alter with time but was influenced by transfusion type, being consistently higher for infants exposed to washed blood compared to unwashed (t=3.72, p<0.01, 95% CI 0.49 to

1.62). No change in percentage of time out of target band, respiratory severity score, or fraction of inspired oxygen with either time or transfusion pack type.

5.5 Discussion

PRBC transfusion should be beneficial through reducing end organ oxygen consumption secondary to improvements in oxygen carrying capacity and therefore resulting in a net improvement in physiological stability [238]. However, there is now an appreciation that it may result in acute adverse physiological responses particularly involving the cardio-respiratory system. It remains challenging to differentiate the perceived need for and consequent receipt of PRBC transfusion, with the possibility of a transfusion reaction in the context of an evolving critical illness. While these unwanted consequences of transfusion are more robustly defined in the adult critical care literature [244], it has been proposed that they may be under-recognised in other vulnerable patient groups, including the extremely preterm infant [245].

Whilst recent attention has focused on TRIM as central to the link between transfusion exposure and adverse outcome in the preterm infant [36], it remains unknown if the potential harmful consequences are due to acute adverse physiological responses, inflammatory processes related to TRIM, or both. This study of the cardio-respiratory responses to first transfusion exposure in extremely preterm infants found that, in addition to changing with time, CO and SVR changed in a transfusion type specific manner, with CO falling and SVR increasing to a greater degree in response to transfusion with washed compared to unwashed PRBCs. In addition, diastolic blood pressure and respiratory rate increased with time following transfusion. The mechanisms underlying this different response to washed and unwashed blood remain open to conjecture.

With respect to the cardiovascular response to transfusion, CO fell, while DBP and SVR increased. Previous studies of transfusion associated effects on cardiac output have produced conflicting results. While the majority of studies have reported a similar fall in CO [250-253], others have reported increases in CO, although this appears to be related to a greater degree of pre-transfusion anaemia as measured by lower haematocrit [120, 183]. As the pre-transfusion haemoglobin concentrations were similar between those infants transfused with unwashed versus washed blood, a different baseline does not explain the greater fall in CO observed following transfusion with washed blood.

Anaemia results in a compensatory increase in CO in an effort to maintain systemic oxygen transport in the preterm infant. RBC transfusion then increases oxygen-carrying capacity by increasing haemoglobin concentration, but also oxygen off-loading to the tissues due to the rightward shift of the oxygen dissociation curve due to transfusion with adult Hb. Washing RBCs has previously been shown to decrease pH in the transfusion pack [254], further shifting the oxygen dissociation curve to the right making it easier for haemoglobin to release bound oxygen and therefore increasing the partial pressure of oxygen in the tissues. If this results in a reduction in end-organ oxygen extraction, as previously described [120, 250], the net result could ultimately manifest as greater fall in the compensatory increase in CO as observed in the current study.

Diastolic blood pressure increased over time; an effect not influenced by transfusion type. DBP represents the resting pressure of blood on the vessels and is reflective of systemic vascular tone/resistance and intravascular blood volume status [255]. Therefore, the increase in DBP is entirely in keeping with an increase in intravascular volume following transfusion in addition to the increase in SVR observed. Initial diastolic hypotension with preserved systolic pressure is common in the early postnatal period and reflects the initial stages where right ventricle (RV)

afterload declines and SVR rises after birth in the face of mild systemic end organ dysfunction [256]. While the presence of a clinically significant PDA commonly complicates this period of early postnatal life, the majority of previous studies have excluded infants with a clinically significant PDA. As this study was conducted during the acute phase of physiological transition following preterm birth, a large proportion in both the washed and unwashed group had a significant PDA. It is likely that the volume of the transductal shunt was sufficiently large that cardiovascular compromise ensued characterised clinically by hypotension and the requirement for inotropic support [256]. With a significant number of infants in the current study receiving inotropes at the time of their first transfusion, the failure to observe transfusion-associated alterations in heart rate or other blood pressure parameters may be due to the desired cardiovascular effects of these pharmacologic agents.

The occurrence of TACO first received attention in the 1930s [257], although it was not until the 1990s that renewed attention resulted in it being recognised as a distinct clinical entity [258]. The estimated frequency of TACO varies from 1% in hemovigilance reports up to 11% in critically ill patients [249, 25], with an estimated incidence between 1.5% and 11% in the paediatric intensive care population [259]. TACO presents with the onset of acute respiratory distress (hypoxemia) within 6 hours of a blood transfusion. The 2011 International Society of Blood Transfusion (ISBT) clinical criteria [260] defines TACO as any 4 of the following occurring within 6 hours of completion of transfusion: acute respiratory distress; tachycardia; elevated blood pressure; acute or worsening pulmonary oedema on chest x-ray; and evidence of positive fluid balance. Even without chest x-ray evidence, the current data does not support adverse cardiovascular consequences of transfusion consistent with TACO in this population of extremely preterm infants.

Few studies specifically report the role of transfusion exposure and acute changes in respiratory stability in the newborn infant. Grev and colleagues have reported a retrospective cohort study of extremely low birth weight infants with acute respiratory decompensation defined as ≥1 of the following: (1) ≥10% increase in fraction of inspired oxygen from highest baseline, (2) ≥2 cm H_2O increase from highest baseline in mean airway pressure, or (3) escalation in mode of respiratory support [79]. No difference in the frequency of acute respiratory decompensation was seen between the control and transfusion time periods for 110 PRBC transfusions in 25 infants. In the current study, with a much larger sample size, we observed an increase in respiratory rate with time, an effect not influenced by transfusion type. An increase in respiratory rate following transfusion has been previously reported in infants transfused at higher haematocrits [119]. It remains unclear if this response is due to alterations in pulmonary blood flow following transfusion or inflammatory processes as seen in TRALI secondary to TRIM [119].

We also observed that MAP was consistently higher for infants exposed to washed blood compared to unwashed, although it did not alter over time. One explanation for this consistently higher MAP is that the infants in the washed group had more severe early neonatal lung disease. However, there were no differences in baseline clinical characteristics that might influence early respiratory function, such as gestational age, birthweight, or the proportion who received antenatal betamethasone. However, while there was no difference in other measures of respiratory illness between the groups, such as FiO_2 or respiratory severity score, more were mechanically ventilated at the time of first transfusion although this did not reach statistical significance.

Acute respiratory distress syndrome, of which TRALI is a subgroup, was first described clinically in 1967 [261]. Concerns regarding the application of this diagnosis to paediatric patients prompted the paediatric acute respiratory syndrome definition [262] which still excluded the first

month of life. This finally resulted in further refinement led to the Montreux definition [263] based on timing, lung imaging, absence of congenital heart disease including a patent ductus arteriosus, and an oxygen deficit measured by an oxygenation index [263]. Over time, the accepted definition of TRALI has evolved, with the current working definition; "Acute dyspnoea with hypoxia and pulmonary infiltrates within 6 hours of transfusion, with no other apparent cause" [264].

The 10-year prospective study of serious transfusion related complications in the United Kingdom [202] has provided the most detailed data on reported adverse events in children with a pooled epidemiologic estimate of adverse outcome rates in 18:100 000 red cell transfusions for children <18 years of age and an overall incidence of 37 000 for infants <1 month of age [265]. While rare, rates of TRALI were 6% which, not-withstanding its relative rarity, does support it being a significant cause of transfusion-related mortality. The current data, as for TACO, fails to support transfusion associated acute respiratory decompensation consistent with TRALI as defined in other patient populations in the extremely preterm infant. The SHOT childhood study authors themselves concluded that "transfusion-related acute lung injury is difficult to diagnose in any patient and may pose particular diagnostic problems in children" [265]. Equally important is the concern that an unreliable definition for TRALI, especially for specific patient populations, may draw false conclusions about its risk factors and biology [266].

TRALI, in particular, has been proposed to have an inflammatory aetiology with a temporal relationship to alternative risk factors for acute respiratory distress syndrome such as sepsis or pneumonia central to its development [267]. Biomarkers have increasingly been utilised in clinical diagnosis and decision making. For example, the use of biomarkers such as troponin and brain natriuretic peptide (BNP) has been investigated in the setting of transfusion-related adverse outcomes although any additional diagnostic value appears limited [268]. More recently

inflammatory biomarkers have been found to predict the onset and underlying pathogenesis of nonhydrostatic (e.g., permeability) pulmonary oedema [269, 270]. Importantly, pre-transfusion and post-transfusion alterations in these inflammatory cytokines have also been recognised in patients who develop TRALI and TACO [271-273]. However, the association between post-transfusion plasma pro-inflammatory cytokine concentrations and alterations in clinical markers of cardio-respiratory function in the extremely preterm infant has not been reported.

There are a number of limitations to the current study. It is important to note that the physiological data was only collected for the first transfusion exposure. This is an important consideration given that numerous studies have demonstrated immunomodulation following exposure to 3 to 4 PRBC transfusions [49, 50, 166, 168]. While there is evidence for alterations in pro-inflammatory cytokine production and endothelial activation in response to single transfusion exposure [63], no studies have specifically investigated if repeat transfusion exposure results in similar adverse responses. There is, however, circumstantial evidence that repeat transfusion may indeed lead to endothelial activation and increased morbidity with an association between repeat PRBC transfusion exposure and increased ischemic damage reported in adult cardiac patients [168]. Therefore, it is possible that the magnitude of any physiological response to transfusion may increase with repeat transfusion exposure if TRIM is a significant contributor to the underlying pathophysiologic processes. Further, measuring post-transfusion cytokine concentrations at a single time point does not capture differences in the timing of peak cytokine responses known to occur after an inflammatory stimulus such as transfusion exposure. However, we were constrained within the design of the current study with respect to the number and timing of blood sampling in this vulnerable patient group. Similarly, we could not perform a chest x-ray following transfusion exposure, as required for the current diagnostic criteria for TRALI and TACO, without a clear clinical indication. Finally, given the very high incidence of respiratory and cardiovascular instability in this patient group, with evolving

clinical signs and symptoms, it may simply be impossible to delineate additional physiologic burden imposed by transfusion exposure.

In summary, the current data focusing on the first transfusion exposure in the extremely preterm infant supports both changes over time and in response to the type of PRBCs transfused. However, the alterations in CO, SVR, DBP, MAP and respiratory rate do not meet the current accepted definitions for either TRALI or TACO employed in other critically ill populations and while statistically significant their clinical significance remains unknown. This is likely due to the unique clinical presentation of the extremely preterm infant. Specifically, transfusion-related alterations in cardio-respiratory stability may be masked by the evolution of significant morbidities such as neonatal respiratory distress syndrome and cardiovascular instability secondary to poor cardiac function and the influence of a significant PDA which complicate the early postnatal period. Interestingly, we observed an association between transfusion-related changes in the pro-inflammatory cytokine IL-6 and CO and SVR, despite a failure to meet TACO or TRALI diagnostic definitions. Therefore, while the potential harmful consequences of transfusion secondary to acute adverse physiological responses analogous with TACO and TRALI cannot be ruled out, these findings suggest a potential for an interaction between transfusion-related immunomodulation and organ dysfunction which warrants further investigation. Whether any adverse effect is modifiable through an alteration in RBC processing, such as red cell washing, deserves specific attention.

Chapter 6

Transfusion Related Changes in Pro- and Anti-Inflammatory Cytokines and Markers of Endothelial Activation

6.1 Introduction

The proposed mechanisms underlying the association between PRBC and the spectrum of post-transfusion multi-organ morbidity and mortality, remain poorly understood. One potential contributing pathway may be the transfusion recipient's immune response to the transfusion itself. Numerous pre-clinical studies demonstrate that RBC products can directly modulate immune cell function [59]. For instance, leukodepleted PRBCs have been shown to prime mononuclear cells resulting in significant increases in pro-inflammatory cytokines [104]. This effect has been termed TRIM and describes both adverse pro-inflammatory and immunosuppressive responses.

TRIM has been proposed to be a "two-insult" process [97]. Initial sensitisation to inflammatory processes results in priming of host neutrophils (first insult). Applying this model to the preterm infant, the first 'hit' may be the pathophysiologic process resulting in preterm birth. Subsequent exposure to biological response mediators passively transfused along with the PRBCs then results in a pro-inflammatory immune response (second insult) [98]. While these pathological processes are yet to be fully characterised, biological response mediators that may be implicated in the second "hit" include donor antibodies, bioactive lipids, free haemoglobin, red cell membrane fragments and cytokines that accumulate during blood product storage [95].

In vitro data supports the ability of the supernatant from stored PRBCs to "prime" unstimulated allogeneic neutrophils, inducing the release of IL-8 and altering the cells chemotactic properties [99]. Further, TRIM appears to be characterised by activation of vascular endothelial cells and platelets. These cells are highly sensitive to inflammatory signals and may release toxic bioactive mediators following activation by donor blood [98]. While TRIM has not been definitively proven in preterm infants, this patient population are potentially at the greatest risk of immunomodulation due to their immature immune system. As a result, it may represent

one of the central processes underlying TRALI; associated with an increase in BPD and TRAGI; linked to an increase in the occurrence of NEC [95], morbidities characterised by inflammation and endothelial activation [274, 275].

While modifications in red blood cell processing, such as pre-storage leukodepletion, have resulted in reduction in the incidence of common neonatal morbidities such as ROP, BPD, and NEC, as well as a trend towards a reduction in bacteraemia and IVH [129], they have not completely abrogated the immunomodulatory effect of PRBC transfusions. Specifically, transfusion with leukodepleted PRBCs is still associated with post-transfusion increases in pro-inflammatory cytokines (IL-1ß, IL-8, TNF) and markers of endothelial activation [63 37]. Recent pre-clinical evidence, however, has suggested that pre-transfusion washing of PRBCs removes proteins, extracellular potassium, inflammatory cytokines and chemokines, as well as RBC microparticles [172], reducing the immunomodulatory potential. Therefore, the aim of this chapter was to investigate whether transfusion with washed leukodepleted red blood cells in the extremely preterm infant would result in an ameliorated post-transfusion pro-inflammatory cytokine response and reduced endothelial activation compared to transfusion with standard, unwashed leukodepleted PRBCs.

6.2 Methods

Parents of extremely preterm infants <29 weeks' gestation were approached and consented prior to their child's first elective transfusion. Randomisation was completed by the SA Pathology transfusion laboratory, with each infant being randomised to either unwashed PRBCs or washed PRBCs for their first and all subsequent PRBC transfusions they required during their primary NICU admission. Within each study arm, infants were stratified by gestational age, 23^{+0} to 25^{+6} and 26^{+0} to 28^{+6} weeks. All transfusions used leukodepleted, O Rh negative PRBCs, with a fixed volume of 15mL/kg transfused over 3 hours. The Australian Red Cross Blood Service

washed PRBC packs 3 days post collection as per standard processing protocols. The SA Pathology transfusion laboratory were unblinded to pack allocation to comply with all other current ordering and safety protocols. However, study investigators, attending clinicians, nursing staff and parents were blinded to product allocation.

As described in Chapter 3, prior to the transfusion, infants had 0.4 mL whole blood collected, with a post-transfusion sample collected 4-6 hours following transfusion completion. A 1 mL sample was also collected from the red cell pack. Samples were centrifuged and plasma aliquoted for analysis of cytokines and markers of endothelial activation analysis. Plasma cytokine levels for each transfusion sample were analysed using the Human High Sensitivity T-Cell Magnetic Bead panel multiplex ELISA (see 3.4.1 High Sensitivity T-Cell Multiplex Assay). Markers of endothelial activation were measured using human sepsis bead panel multiplex ELISA (see 3.4.2 Neonatal Sepsis Assay).

6.2.1 Statistical Analysis

Extremely preterm infants will have a median number of 3-5 transfusion exposures during their primary hospital admission. Therefore, we made an a priori decision to determine transfusion associated changes in circulating cytokines and markers of endothelial activation for the first 4 transfusions in our population of 154 extremely preterm infants. Clinical characteristics are presented as median (IQR) or number (%) where appropriate. Demographic data were compared using ANOVA or Mann-Whitney U depending on distribution and differences in frequencies for categorical variables tested using the χ^2 test or Fisher's Exact.

As the plasma cytokine concentrations were not normally distributed, the Friedman test was used to assess differences between baseline cytokine concentrations prior to each transfusion exposure, with the alpha level set at 0.01 to adjust for multiple comparisons. Pre- and post-transfusion levels of plasma cytokines are presented as median (interquartile range), with

differences assessed using Wilcoxon Signed Rank test with a p value <0.05 considered significant. Within subject temporal changes, in the pre- to post-transfusion Δchange in plasma cytokines and markers of endothelial activation, in each group across the three transfusion exposures were analysed using a Linear Mixed Model. Heterogenous compound symmetry was used as the repeated covariance type and LSD as the confidence interval adjustment, to compare changes within treatment groups. To investigate whether transfusion related changes in cytokines and markers of endothelial activation were influenced by gestational age, sex, age at first transfusion, and pre-transfusion haemoglobin, these variables were included as co-variates. Data were analysed using the Statistical Package for the Social Sciences (SPSS v26; IBM SPSS, Chicago, IL).

6.3 Results

Baseline clinical characteristics are shown in Table 6.1. No differences were seen for gestational age at birth, sex, birth weight or other common antenatal characteristics across transfusion exposure. Postnatal age at transfusion, pre-transfusion Hb and Hb response to transfusion were similar between the unwashed and washed groups for each.

Transfusion pack concentrations of cytokines and markers or endothelial activation

A random selection of transfusion pack samples across the four transfusion exposures were selected (n=36 per study group) to determine if detectable levels of cytokines and markers of endothelial activation were present before transfusion into the recipient. IFNγ, IL-1b, IL-10, IL-12, and IL-17A were undetectable in both the unwashed and washed packs. While IL-6 was detectable in the unwashed packs (median 1.46 (IQR 0.8-7.3) pg/ml), it was undetectable in the washed packs. IL-8 and TNF were present in detectable concentrations in both the unwashed and washed packs, with levels not significantly different (Fig 6.1A and 6.1B). Neither sFas or sFasL were detectable in the unwashed or washed packs. While sICAM1 was only detectable in

11% of the washed packs, it was present in a significantly greater proportion of the unwashed packs (92%, π^2=55.0, p=0.01), with a median concentration of 83.4 (52.9-116.2) pg/ml. Similarly, sVCAM1 and PAI1 were detectable in more unwashed packs than washed PRBC packs, although this difference did not reach significance. However, the detectable concentrations in the unwashed packs were significantly higher than in the washed packs (p<0.001 for both, Fig 6.1C and 6.1D). MIF was detectable in all packs, with levels higher in the washed compared to the unwashed packs (p=0.007, Fig 6.1E).

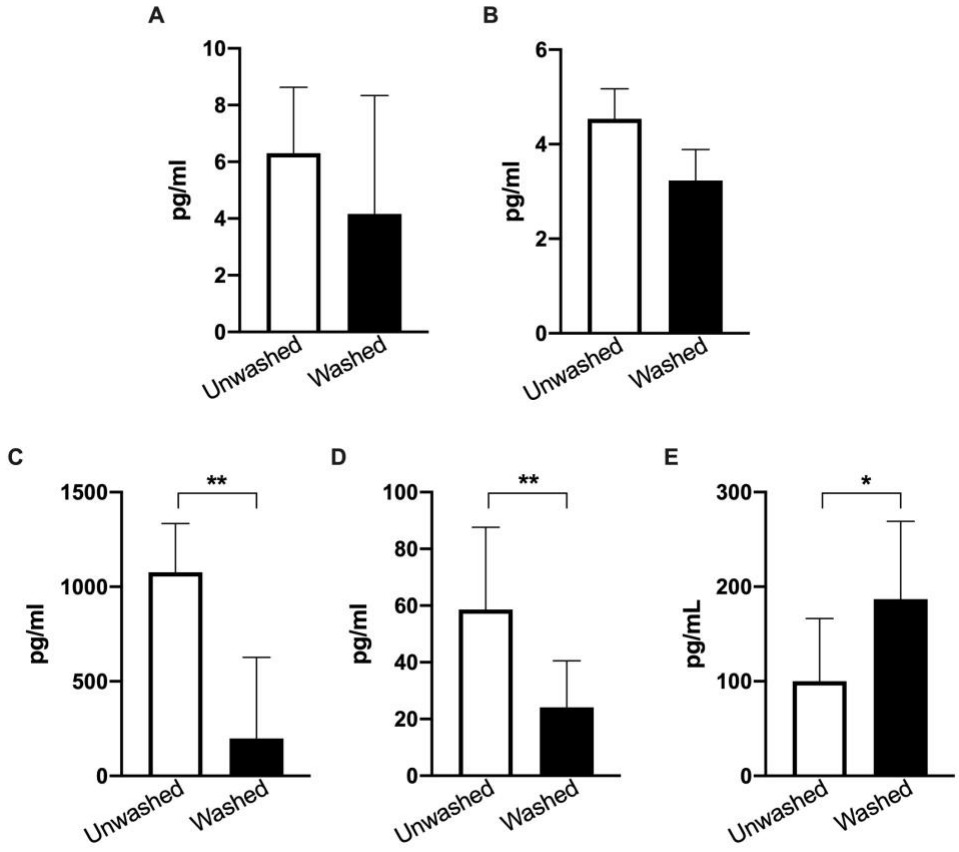

Figure 6.1 Plasma cytokines and markers of endothelial activation within the PRBC pack. A IL-8, B TNF, C sVCAM1, D PAI1, and E MIF. Median (IQR) *p<0.05 **p<0.01.

Table 6.1 Antenatal characteristics across transfusion exposure

	Transfusion 1 (n= 154)		Transfusion 2 (n= 121)		Transfusion 3 (n= 97)		Transfusion 4 (n= 75)	
	UW (n= 77)	W (n= 77)	UW (n= 59)	W (n= 62)	UW (n= 47)	W (n= 50)	UW (n= 37)	W (n= 38)
Gestation	26 (24-27)	26 (25-27)	25 (24-27)	26 (25-27)	25 (24-27)	25 (24-26)	25 (24-27)	25 (24-26)
Sex								
Male	50 (65)	47 (61)	38 (64)	39 (63)	28 (60)	34 (68)	25 (68)	24 (63)
Female	27 (49)	30 (39)	21 (36)	23 (37)	19 (40)	16 (32)	12 (32)	14 (37)
Birthweight	800 (660-966)	830 (650-990)	750 (640-910)	780 (624-950)	692 (630-860)	768 (610-920)	670 (630-770)	703 (590-790)
IUGR	9 (12)	15 (19)	6 (10)	13 (21)	4 (9)	10 (20)	4 (11)	9 (24)
Multiple Birth	22 (29)	17 (22)	13 (22)	8 (13)	11 (23)	5 (10)	10 (27)	4 (11)
Antenatal Steroids								
None	6 (8)	5 (6)	5 (8)	5 (8)	4 (9)	4 (8)	3 (8)	3 (8)
Incomplete	13 (17)	15 (19)	10 (17)	12 (19)	9 (19)	12 (24)	9 (24)	12 (32)
Complete	57 (74)	56 (73)	44 (75)	45 (73)	34 (72)	34 (68)	25 (68)	23 (61)
Chorioamnionitis	30 (39)	23 (30)	26 (44)	21 (34)	21 (45)	16 (32)	17 (46)	12 (32)
Magnesium Sulphate	48 (62)	52 (68)	38 (64)	41 (66)	30 (64)	32 (64)	24 (65)	25 (66)
Mode of Delivery								
SVD	29 (38)	26 (34)	24 (41)	21 (34)	20 (43)	19 (38)	14 (38)	14 (37)
LSCS	48 (62)	51 (66)	35 (59)	41 (66)	27 (57)	31 (62)	23 (62)	24 (63)

Data presented as median (IQR) or N (%). Fisher's Exact Test or Pearson Chi-Square used for comparison between groups. UW- unwashed PRBCs, W- washed PRBCs.

The baseline concentrations of cytokines and markers of endothelial activation in plasma of newborn infants in the unwashed and washed PRBC treatment groups at transfusion 1, 2, 3 and 4 are shown in Table 6.2 and 6.3. Baseline concentrations were compared prior to the 1st, 2nd, 3rd and 4th transfusions, both within group and between the unwashed and washed groups. For those infants receiving unwashed blood, the pre-transfusion cytokine concentrations did not vary between the 1st and 4th transfusion exposures. However, for infants transfused with washed PRBCs, significant overall changes in baseline cytokine concentrations were seen for IFNγ ($p<0.001$), IL-12 ($p=0.012$), IL-17A ($p=0.002$) and IL-1β ($p=0.008$). Post-hoc tests demonstrated increases in baseline IFNγ concentrations from transfusion 1 to 3 ($p=0.002$) and 1 to 4 ($p=0.004$), while IL-12 and IL-17A baseline concentrations increased between transfusion 1 and 3 alone ($p<0.001$ and $p=0.01$ respectively). Pre-transfusion concentrations of markers of endothelial activation did not differ between the 1st and 4th transfusion for either the unwashed or washed groups.

No baseline differences were seen in cytokine concentrations or endothelial markers between the unwashed and washed groups for the first transfusion exposure. However, infants receiving washed PRBCs had increased concentrations of IL-1β ($p=0.03$), IL-6 ($p=0.01$), IFNγ ($p=0.05$), in addition to increased concentrations of MIF ($p=0.004$) and sFas ($p=0.05$) prior to their second transfusion when compared to those infants in the unwashed group. Likewise, infants in the washed group had higher concentrations of IL-1β ($p=0.03$) and IL-6 ($p=0.01$) prior to the third transfusion exposure.

Table 6.2 Baseline cytokine concentrations (pg/ml)

	Transfusion 1		Transfusion 2		Transfusion 3		Transfusion 4	
	UW	W	UW	W	UW	W	UW	W
IFNγ	0.9 (0 – 13.9)	1.32 (0 – 4.9)	0 (0 – 15.2)	4.2* (0.4 – 6.7)	2.58 (0 – 15.4)	5.5 (0.8 – 19)	2.2 (0-12.4)	9.7 (0.9-30.7)
IL-10	13 (1.4 – 40.1)	16 (4 – 32.1)	11.9 (1.7 – 66.3)	12.8 (3.6 – 49.8)	11.3 (1.7 – 25.1)	12 (5.1 – 25.1)	12.3 (0-39.3)	15.3 (5.5-51.3)
IL-12	1.8 (0 – 8.8)	1.7 (0.8 – 6.4)	3.5 (0 – 9)	2.7 (1 – 8)	2.7 (0 – 6.3)	4 (1.3 – 12.5)	5.0 (1.2-11.0)	3.8 (1.1-19.9)
IL-17A	3.5 (0 – 14.8)	2.1 (0.1 – 7)	5.1 (0 – 11.9)	3.4 (0.4 – 9.6)	7.4 (1.2 – 16.7)	5.9 (0.6 – 22)	6.3 (0-13.2)	5.2 (0.25-24.7)
IL-1β	0 (0 – 6.4)	0.5 (0 – 2.8)	0 (0 – 1.2)	0.8* (0 – 5.4)	0 (0 – 2.1)	1.2* (0.3 – 5.3)	0.44 (0-2.3)	1.3 (0.1-9.5)
IL-6	17.58 (0 – 54.6)	28.1 (6.4 – 103)	10.9 (0 – 42.8)	29.9** (7.4 – 87.2)	8.2 (0 – 29.3)	23.8** (8.9 – 73.4)	28.4 (0.1-100.9)	20.4 (4.8-50.3)
IL-8	191 (64 – 314)	252 (118 – 527)	75.4 (37.5 – 242.5)	259.6 (97.5 – 455)	89.2 (35.3 – 274.4)	285.3 (67.4 – 396.2)	244.3 (132-414.3)	280.5 (59.5-792.6)
TNF	50.2 (31.8 – 81)	40.2 (28.5 – 66)	40.8 (26 – 93.9)	44.2 (30.2 – 71.6)	46.5 (29.9 – 66.9)	55.5 (32.7 – 73.6)	60.4 (33.4-86.5)	51.8 (33.5-86.5)

Data presented as median (IQR). UW – unwashed PRBCs, W – washed PRBCs. Mann-Whitney U used to compare unwashed vs washed.
*p<0.05 **p<0.01

Table 6.3 Baseline concentrations of markers of endothelial activation (pg/ml unless otherwise stated)

	Transfusion 1		Transfusion 2		Transfusion 3		Transfusion 4	
	UW	W	UW	W	UW	W	UW	W
MIF	126.5	149	121.8	237.3**	132.8	126.8	200.4	268.3
	(61.7 – 296.2)	(62 – 349)	(98.9 – 210.2)	(126.5 – 628.2)	(70.2 – 332.9)	(50.8 – 404.9)	(90.1-392.9)	(77.1-416-9)
sICAM1 (ng/ml)	19.8	20.4	22.9	26.4	29.0	23.6	30.3	38.1
	(14.3 – 31.4)	(10.9 – 32.6)	(16.0 – 34.7)	(13.3 – 41.7)	(23.8 – 42.9)	(11.5 – 32.9)	(18.1-65.0)	(31.6-54.1)
sFasL	17.2	15.1	15.4	17.4	15.2	18.9	14.9	22.4
	(6.7 – 28.7)	(8.6 – 30.6)	(7.1 – 25.6)	(11.3 – 37.2)	(8.5 – 26.5)	(5.2 – 33.4)	(10.6-48.2)	(8.5-60.0)
sFas	245	322	257.8	343*	247	318	421	375
	(173 – 380)	(261 – 442)	(168.7 – 392)	(242 – 439)	(194 – 506)	(221 – 422)	(260.9-538-3)	(199-685)
sVCAM1 (ng/ml)	227	226	251	246	190	259	287	245
	(171 – 389)	(19 – 446)	(160 – 367)	(184 – 420)	(113 – 375)	(114 – 397)	(158-504)	(163-366)
PAI1 (ng/ml)	12.2	9.3	9.5	14.1	6.9	10.5	11.8	10.8
	(7.0 – 16.6)	(4.2 – 17.1)	(4.3 – 13.0)	(4.1 – 25.5)	(3.8 – 13.9)	(7.6 – 17.5)	(5.9-22.4)	(5.9-24.4)

Data presented as median (IQR). UW – unwashed PRBCs, W – washed PRBCs. Mann-Whitney U used to compare unwashed vs washed. *p<0.05 **p<0.01

Transfusion associated changes in plasma cytokine and markers of endothelial activation

Neither unwashed or washed PRBCs resulted in significant post-transfusion changes in IL-1β, IL-12, IL-17A, IFNγ or TNF at the first transfusion exposure. However, transfusion with washed PRBCs was associated with a significant fall in post-transfusion IL-6 (p=0.008, Figure 6.2A) and IL-10 (p=0.014, Figure 6.2B), with transfusion of both unwashed and washed PRBCs resulting in a reduction in circulating IL-8 (unwashed p=0.024, washed p=0.027, Figure 6.2C). No changes were observed for any markers of endothelial activation.

At the second transfusion exposure, no post-transfusion alterations were seen for IL-1β, IL-6 and IL-17A for either unwashed or washed PRBCs. However, transfusion with washed PRBCs resulted in a reduction in IFNγ (p=0.043, Figure 6.3A), TNF (p=0.041, Figure 6.3B), IL-8 (p=0.004, Figure 6.3C), and IL-10 (p=0.003, Figure 6.3D) and an increase in IL-12 (p=0.01, Figure 6.3E). No change was observed for MIF, sVCAM1, sFasL and PAI1. However, transfusion with unwashed PRBCs was associated with an increase in sICAM1 (p=0.023, Figure 6.4A) with transfusion with washed PRBCs associated with an increase in sFas (p=0.026, Figure 6.4B)

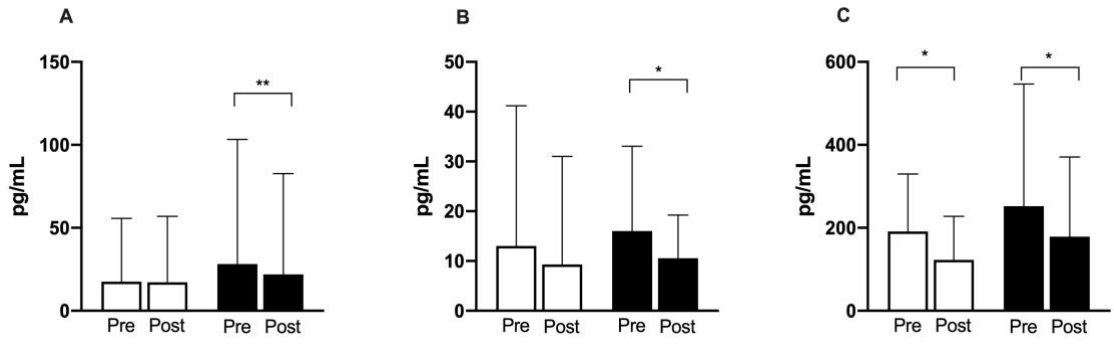

Figure 6.2 Plasma cytokine response to 1st transfusion exposure (□ unwashed■ washed). A IL-6, B IL-10 and C IL-8. Median (IQR) *p<0.05**p<0.01.

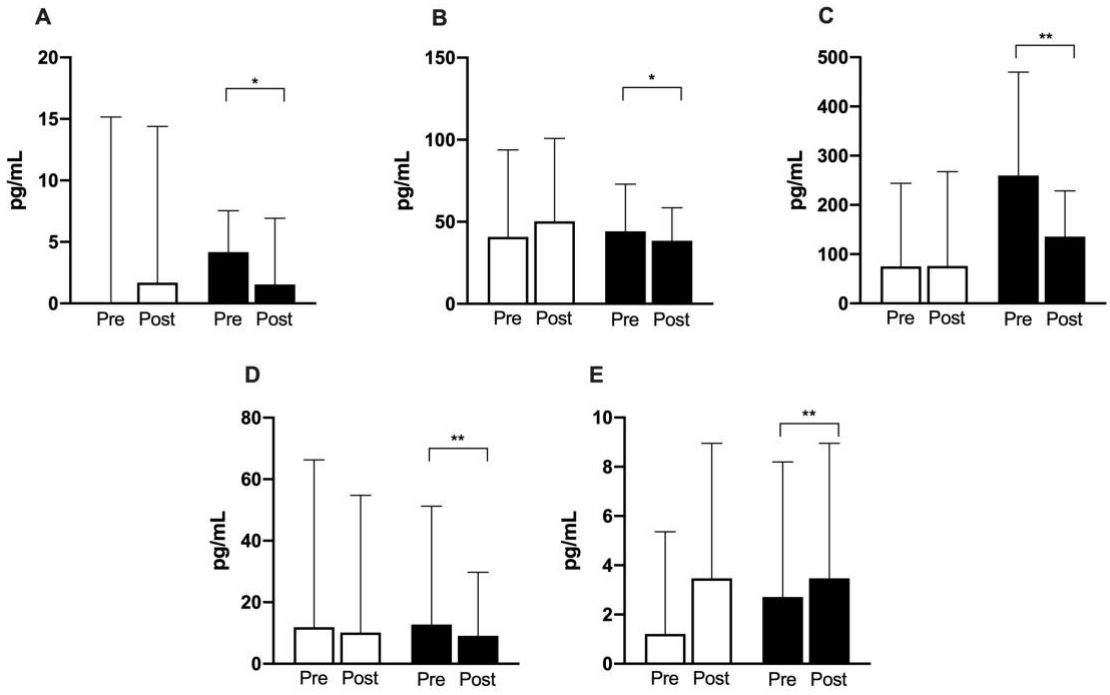

Figure 6.3 Plasma cytokine response to 2nd transfusion exposure (☐ unwashed ■ washed). A IFNγ, B TNF, C IL-8, D IL-10 and E IL-12. Median (IQR) *p<0.05 **p<0.01.

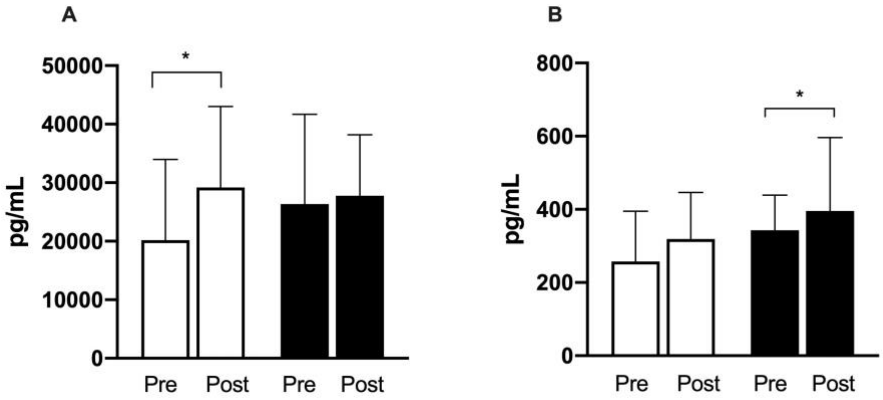

Figure 6.4 Markers of endothelial activation response to 2nd transfusion (☐unwashed■washed). A sICAM1 and B sFas. Median (IQR) *p<0.05 **p<0.01.

By the 3rd transfusion significant post-transfusion increases were seen for IL-17A (p=0.05) and TNF (p=0.007) in those infants transfused with unwashed blood. Conversely, transfusion with washed PRBCs was associated with a decrease in IL-17A (p=0.013, Figure 6.5A), TNF (p=0.05, Figure 6.5B), IL-6 (p=0.001, Figure 6.5C), IL-8 (p=0.037, Figure 6.5D), IL-12 (p=0.001, Figure 6.5E) and IFNγ (p=0.001, Figure 6.5F). No transfusion-related changes were seen for markers of endothelial activation for those infants receiving washed blood. However, in those

transfused with unwashed PRBCs, there was an increase in MIF (p=0.005, Figure 6.6A) and PAI1 (p=0.05, Figure 6.6B). Finally, no differences were seen for either unwashed or washed PRBCs at the fourth transfusion exposure for any of the circulating cytokines or markers of endothelial activation.

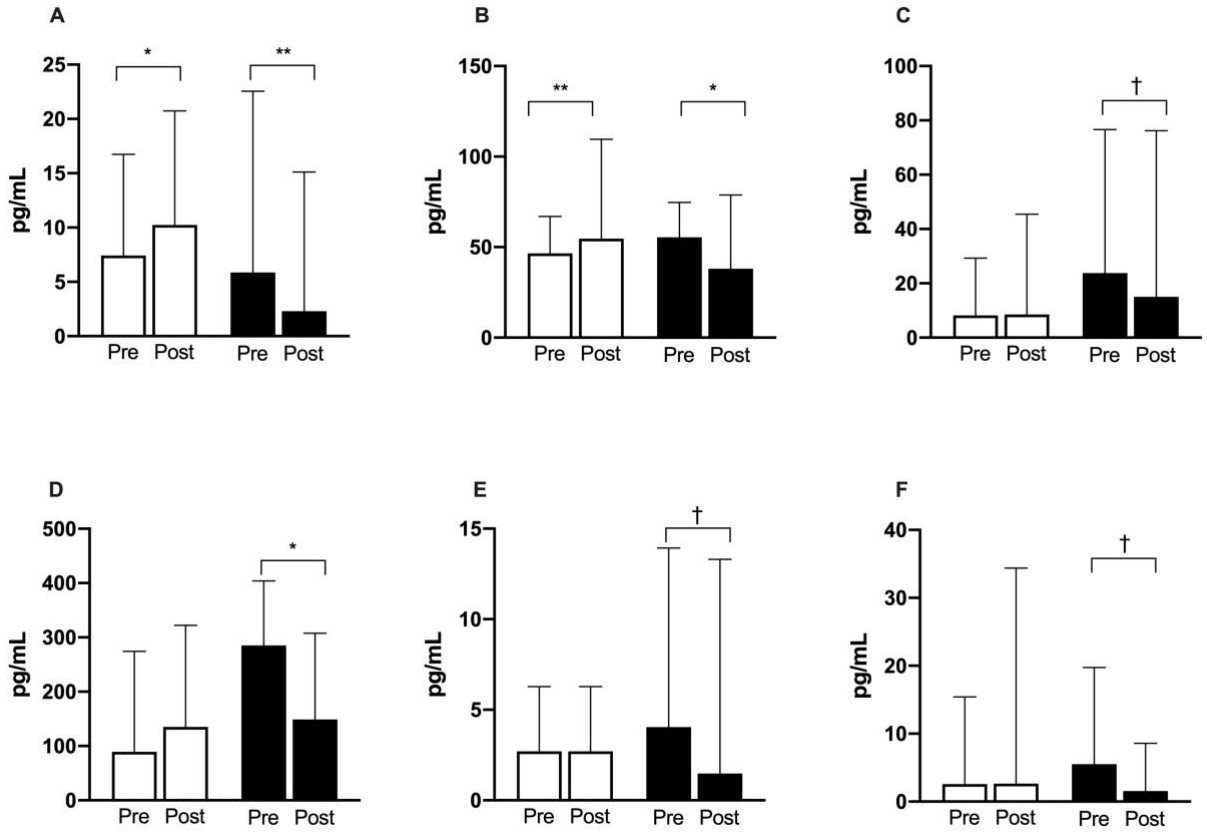

Figure 6.5 Plasma cytokine response to 3rd transfusion exposure (□unwashed ■washed). A IL-17A, B TNF, C IL-6, D IL-8, E IL-12 and F IFNγ. Median (IQR) *p<0.05 **p<0.01 †p<0.001.

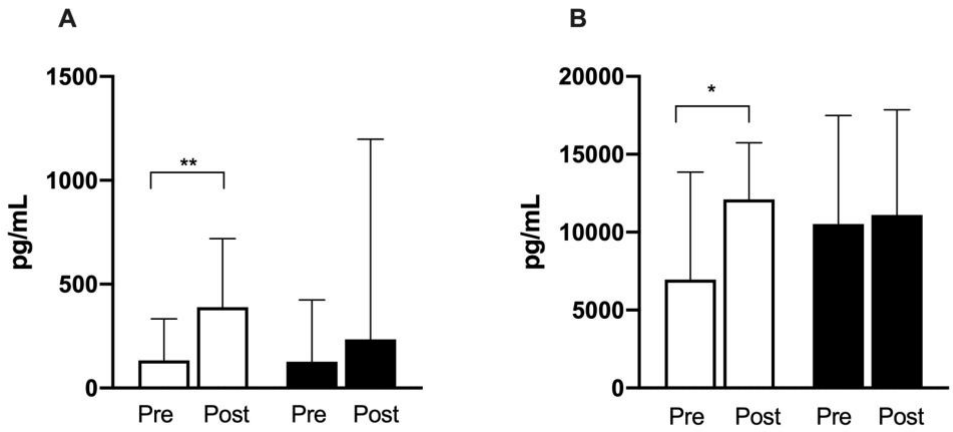

Figure 6.6 Markers of endothelial activation response to 3rd transfusion(□unwashed■ washed). A MIF and B PAI1. Median (IQR) *p<0.05 **p<0.01.

Differential effect of transfusion with unwashed versus washed PRBCs on plasma cytokines

Within subject temporal changes in the pre- to post-transfusion Δchanges in plasma cytokines and markers of endothelial activation in each group across the three transfusion exposures were analysed using a Linear Mixed Model with gestational age, sex, age at first transfusion, and pre-transfusion haemoglobin included as co-variates. As the number of infants who went on to receive a 4^{th} transfusion was significantly reduced in both the unwashed and washed PRBC groups this analysis was confined to the first three transfusion exposures.

Table 6.4 Fixed effects values for transfusion exposure

Cytokine	Pack-type x transfusion exposure
IL12	($F_{(1, 307)}$= 4.0, p=0.04)
IL17A	($F_{(1, 307)}$= 4.9, p=0.03)
TNF	($F_{(1, 206)}$= 2.4, p=0.046)

Tests for fixed effects demonstrated a significant interaction effect between PRBC transfusion pack type and transfusion exposure for IL12, IL17A, and TNF (Table 6.4). For infants transfused with washed PRBCs, the post-transfusion Δchange in fell between the 3 transfusion exposures for IL12 ($F_{(1, 151)}$= 10.5, p=0.001), IL17A ($F_{(1, 151)}$= 6.9, p=0.01) and TNF ($F_{(1, 156)}$= 4.4, p=0.03) while no change in the magnitude of the transfusion related change was observed in infants transfused with unwashed blood (Fig 6.7 A, B and C). Further, a fixed effect for transfusion exposure was demonstrated for IFNγ ($F_{(1, 236)}$= 4.9, p=0.028) with the Δchange greatest after the third transfusion irrespective of the type of PRBCs transfused.

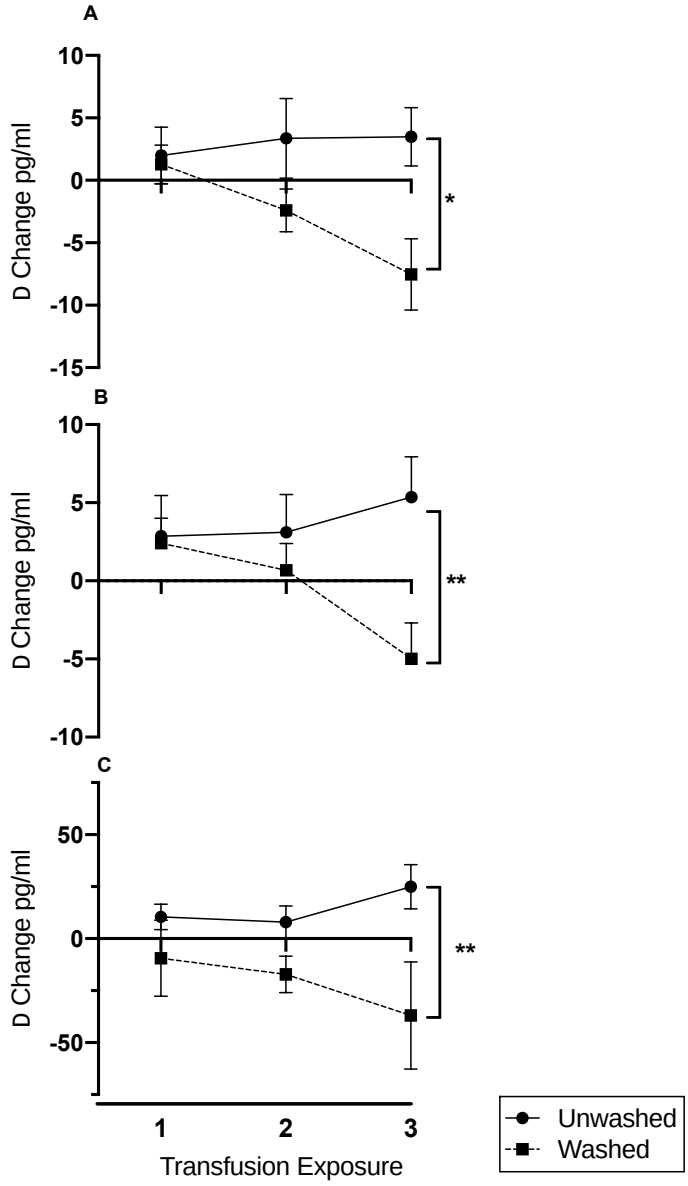

Figure 6.7 Mean (SEM) change in cytokine concentrations with repeated transfusion exposure. A IL-12, B IL-17A and C TNF. *p<0.05 **p<0.01

6.4 Discussion

There is a paucity of studies addressing potential mechanistic pathways underlying the association between PRBC transfusion exposure and adverse neonatal outcome. The most recent meta-analysis of the literature focusing on neonatal transfusion and adverse outcome has recognised this as a clear knowledge gap [60]. Recently, attention has shifted to investigating the recipient's inflammatory response to transfusion. These studies report increases in circulating plasma concentrations of pro-inflammatory cytokines and markers of endothelial activation from 2-48 hours after a single transfusion exposure in the weeks following preterm

birth [37, 63]. This inflammatory response occurs despite modifications to PRBC processing such as leukodepletion which are proposed to reduce their inflammatory potential and therefore the incidence of transfusion associated neonatal morbidity and mortality [129].

There are even fewer studies addressing the potential for transfusion with washed leukodepleted PRBCs to further ameliorate this unwanted consequence of transfusion exposure in the critically ill. In the current study, transfusion with washed PRBCs resulted in significant post-transfusion decreases in plasma pro-inflammatory cytokines and chemokines, while transfusion with unwashed PRBCs resulted in the opposite, with significant increases in IL-17A and TNF. By the 3rd transfusion exposure this response to unwashed blood was also associated with an increase in markers of endothelial activation, specifically MIF and PAI1, an effect not seen with washed PRBCs. This data suggests that not only is the potential for transfusion-related immunomodulation present in the initial days following extremely preterm birth, changes with on-going repeated transfusion exposure and critically can be modified by transfusion with washed PRBCs. If transfusion-related immunomodulation does constitute a critical process linking PRBC exposure to morbidity and mortality in this high-risk patient group, the current data suggests that the use of washed PRBCs could reduce the adverse impact of TRIM and ultimately result in improved clinical outcomes.

Extremely preterm infants represent a particularly heavily transfused population receiving on average 3-5 blood transfusions during their hospital admission most commonly early in their admission to NICU [48]. Despite this, previous studies have focused on single exposures often weeks after preterm birth [37, 63]. A strength of the current study was that repeated transfusion exposure rather than a single transfusion event was investigated. For infants transfused with washed PRBCs lower post-transfusion plasma concentrations of pro-inflammatory cytokines were seen after the 1st, 2nd, and 3rd transfusion exposures, an effect not observed with

unwashed PRBCs. The most striking difference in response to unwashed and washed PRBCs was observed following the 3rd transfusion, specifically opposite effects on plasma concentrations of IL-12, IL-17A and TNF, with transfusion with washed PRBCs eliciting a significantly reduced pro-inflammatory response. This appears to be independent of gestational age, sex, and degree of pre-transfusion anaemia. The pro-inflammatory response to repeat transfusion exposure with unwashed PRBCs is analogous with observational data suggesting a relationship between increasing transfusion exposure and/or cumulative volume of blood transfused and risk of overall mortality [170] and morbidities such as BPD, ROP, and NEC [193, 276, 277]. However, whether the observed differences in recipient immune responses between those transfused with washed versus unwashed PRBC translates to clinically meaningful outcomes remains unknown.

The interaction between pro-inflammatory cytokines and the endothelium may play an important role linking transfusion exposure to adverse clinical outcomes. For instance, TNF and IL-1β activate endothelial cells resulting in the recruitment of leukocytes to sites of cellular damage [278] and IL-8 promotes leukocyte emigration from the vasculature [279]. Central to these processes is MIF, which promotes the expression of endothelial P-selectin and the arrest of leukocyte rolling [280]. MIF is important in the regulation of host inflammatory and immune responses and is produced by monocytes/macrophages upon stimulation with various pro-inflammatory stimuli, including TNF and interferon [281]. PRBC type specific alterations in PAI1 were also observed. PAI1 is not commonly associated with the preterm morbidities, however it has recently been proposed to play a role in the pathogenesis of BPD [282]. It is produced by a number of cells such as macrophages, adipocytes and cardiac myocytes and increased expression is associated with tissue and vascular damage [283]. PAI-1 is thought to be part of the acute phase response and is strongly influenced by inflammatory cytokines such as TNF and IL-6. The current data supports a link between TRIM and endothelial activation or damage, with

the 3rd transfusion exposure to unwashed but not washed PRBCs associated with post-transfusion increases in TNF, MIF and PAI1. This transfusion related effect could contribute to organ dysfunction and morbidity, for instance post-transfusion lung injury [78].

While a "two-insult" hypothesis has been proposed to underlie TRIM the exact mechanism/s remains poorly understood. It has been suggested that TRIM results in long term or permanent alteration to immune function [167] which is then compounded by repeat transfusion exposure [50]. While the volume needed to induce TRIM is likely highly patient specific [49], previous studies have demonstrated immunomodulation following exposure to 3-4 PRBC transfusions [49, 50, 166, 168]. This is in agreement with the current data where the most marked transfusion-related changes in cytokines and markers of endothelial activation occurred following the third transfusion exposure. However, the lower post-transfusion cytokine concentrations seen following transfusion with washed PRBCs was surprising. One possible explanation is that anaemia per se is known to be pro-inflammatory with the paediatric and adult literature reporting elevated concentrations of IFNγ, IL-6 and TNF in iron deficiency anaemia [284, 285] and elevated IL-2, IL-6 and TNF in aplastic anaemia [286]. Further, in a murine model of acute blood loss, the resultant pro-inflammatory response is blunted by subsequent transfusion with reductions in IFNγ, IL-6, and IL-10 [287]. However, it is unknown what effect, if any, anaemia of prematurity in the extremely preterm infant has on basal production of pro-inflammatory cytokines. Nonetheless, if similar increases in circulating cytokines did occur, transfusion with washed PRBCs may result in a dampened inflammatory response in the recipient resulting in a net reduction in post-transfusion cytokine concentrations. Transfusion with unwashed PRBCs, resulting in greater transfusion-associated immunomodulation and pro-inflammatory cytokine production, may conversely result in comparable or greater concentrations of post-transfusion circulating cytokines despite correction of the initial pro-inflammatory anaemic state. Interestingly, when degree of pre-transfusion anaemia was included as a co-variate in the

mixed linear models' analysis of the temporal pattern of Δchange in cytokine and markers of endothelial activation concentration following the first 3 transfusion exposures, it did not exert a significant effect.

We know that washing PRBCs alters their immunomodulatory potential [254]. In addition to residual leukocytes, PRBC units also contain non-polar lipids and a mixture of pro-inflammatory lysophosphatidylcholines (lyso-PCs) [288]. Lyso-PC modulates the activity of NKT and T cells [289], acts as an NK cell chemoattractant [290], induces dendritic cell maturation [291], and stimulates the production of pro-inflammatory cytokines [292]. In addition, ecosanoids (prostaglandins and thromboxanes) can also accumulate in PRBCs [293]. PRBC washing removes these bioactive substances along with extracellular potassium, inflammatory cytokines and chemokines, as well as cell-free haemoglobin and PRBC microparticles [172]. These reductions occur not only when packs are washed at the initial storage time but also following storage for a number of days. Importantly, in vivo studies have shown that incubation of pulmonary endothelial cells with supernatant from washed PRBC transfusion packs is not associated with the increased endothelial permeability or pro-inflammatory cytokine and chemokine release observed following incubation with supernatant from unwashed PRBCs [172, 174]. The sole study comparing the effect of transfusion with unwashed PRBCs to washed PRBCs in a paediatric population demonstrates a reduction in the post-transfusion increases in IL-6 and IL-10 in the washed group [163]. The current data suggests that PRBC washing is associated with a similar effect in the transfused extremely preterm infant, with a different response to that observed with unwashed PRBC characterised by less pronounced effect on circulating cytokines and no impact on markers of endothelial activation. This finding suggests the TRIM response is different to washed PRBCs which, in this high risk, heavily transfused population, warrants further detailed investigation linking altered immune responses with subsequent morbidity and mortality.

While baseline pre-transfusion cytokine concentrations did not alter between the 1st and 4th transfusion for those infants who received unwashed PRBCs, pre-transfusion concentrations of IFNγ, IL-1β, IL-12 and IL-17A increased in those who received washed PRBCs. This was not due to differences in clinical characteristics such as postnatal age, degree of anaemia, requirement for invasive respiratory support (as a proxy for severity of lung disease) or proportion receiving antibiotics for suspected or proven sepsis at the time of transfusion exposure, all of which could alter circulating concentrations of pro-inflammatory cytokines. While these findings may reflect the influence of a reduction in the sample size with each subsequent transfusion and inherent biological variability in the recipient's response to PRBC exposure, data on temporal changes in cytokine concentrations in the first weeks of life in preterm infants are very limited [294, 295] and frequently conflicting. For some of the cytokines and chemokines an increase after birth is found, while for others, a decrease is observed [296, 297]. Data from extremely preterm infants is even more limited and confounded by small sample sizes [298], the influence of an increased incidence of intra-uterine exposure to inflammation [299] or a focus on characterising patterns of change in post-natal cytokine concentrations as a predictor of neonatal morbidity such as BPD or brain injury [300]. With TRIM proposed to result in long-term alterations in immune function (ref 167) and compromising both pro-inflammatory and immunosuppressive components, the lack of a post-natal age dependent change in baseline cytokine concentrations in the unwashed PRBC group may represent an aberrant developmental response. Despite unchanged baseline levels the magnitude of the post-transfusion increases in cytokines grew with subsequent transfusion exposure. Conversely, in the washed group, for those cytokines where the baseline increased between the 1st and subsequent transfusions, the post-transfusion concentration progressively fell. Further, while the changes in baseline concentrations in the unwashed group did not reach significance, the pattern of change between the 1st and 4th transfusions were similar. This may reflect the influence of a reduction

in the sample size with each subsequent transfusion and inherent biological variability in the recipient's response to PRBC exposure.

Previous studies, focusing solely on unwashed leukodepleted PRBC packs, have reported conflicting evidence regarding the presence of detectable levels of pro-inflammatory cytokines and markers of endothelial activation in the supernatant of the transfusion units [37, 63, 162]. In the current study, IL-6 was detectable in the unwashed PRBC transfusion packs alone, while IL-8 and TNF were present in comparable concentrations in the supernatant of pack samples from both unwashed and washed PRBCs. Further, the markers of endothelial activation sICAM1, sVCAM1 and PAI1 were detectable in more unwashed PRBC packs than washed PRBC packs, and in higher concentrations. Conversely, while MIF was detectable in both pack types, levels were higher in the washed PRBC units. Firstly, due to methodological constraints, the pack concentrations where only measured in 72 samples selected at random. However, the findings are consistent with those previously reported by Keir and colleagues in unwashed leukodepleted PRBCs used for transfusion in preterm infants [63]. However, they contradict the findings of Dani [37] and Locke [162] who report no detectable cytokine levels. While the age of the PRBCs transfused and the multiplex assay approach employed to measure the concentration of multiple analytes were similar between the current study and that of Dani and colleagues [37], there are significant methodological differences that deserve consideration. The most significant difference is related to the blood pack additive solution used. Studies by Dani and Locke using unwashed PRBCs containing AS-5 Optisol [37, 162]. The PRBC packs in the current study and that of Keir and colleagues contained SAG-M [63]. While some studies have failed to show a difference in cytokine content of PRBC units stored in different solutions [301], others have reported marked differences with the media used having a significant impact on pack cytokine levels [302] and on inherent properties of the stored PRBCs, such as microparticle production and endothelial adhesion, effects greater with SAG-M [303].

Irrespective of the inconsistencies in the published data, the biological significance of the presence of cytokines and markers of endothelial activation in the unwashed and washed PRBC remains unknown. We observed an increase in IL-12 and IL-17A following the second and third transfusions respectively, neither of which were detected in the transfusion packs, post-transfusion concentrations of MIF where greater in the unwashed group despite this group exhibiting lower pack levels. Further, if any increase was solely the result of infusion of cytokines along with the PRBCs, one would expect a consistent post-transfusion change in cytokine concentration following the 1st, 2nd and 3rd transfusions, rather than specific changes that differed.

The current study has a number of strengths and limitations. The requirement for transfusion and age of PRBCs transfused was controlled through the use of a transfusion threshold defined a priori and a maximal shelf life of 14 days. These measures limit the impact of difference in the age of the PRBC pack, therefore storage lesion, and the potential for differences in the degree of anaemia to confound the transfusion related changes in cytokines and markers of endothelial activation. As previously stated, a strength of the current study was that repeated transfusion exposure, rather than a single transfusion event, was investigated. This makes the data more clinically relevant, with extremely preterm infants receiving on average 3-5 blood transfusions during their hospital admission [48]. However, post-transfusion concentrations of cytokines and markers of endothelial activation were measured at a single time point; therefore, it is not known whether these changes were sustained or transient. This is an important consideration, with some data supporting time related changes in specific cytokines and adhesion molecules [37]. Indeed, these changes may be the result of alterations in immune cell phenotype and therefore cytokine production, an effect that can only be conclusively proven through more detailed immune profiling which extends beyond measurement of circulating cytokines and markers of endothelial activation.

While baseline, pre-transfusion cytokine concentrations were higher in the current study in comparison to other studies reporting responses to single transfusion later in the post-natal period [37, 38], this is likely due to samples being collected earlier in the post-natal period. Importantly, the baseline cytokine concentrations observed in the current study are consistent with previous studies characterising the temporal changes in plasma cytokines in extremely preterm infants over the first weeks of postnatal life [298, 300], with the post-transfusion increases observed following exposure to unwashed blood higher than those reported across the neonatal period. However, it is important to acknowledge that temporal changes in plasma cytokines are likely confounded by many other inflammatory signals, both pathological and related to intensive care therapies such as on-going mechanical ventilation experienced by the extremely preterm infant in the first weeks of life [304]. Finally, the number of infants receiving multiple transfusions decreased as transfusion exposure increased. This may have resulted in a loss of power and ability to accurately detect true differences between pre- and post-transfusion concentrations of cytokines and markers of endothelial activation in response to unwashed versus washed PRBC exposure. As a result, the mixed linear models' analysis, which controlled for the contribution of gestational age, sex, postnatal age at first transfusion and degree of pre-transfusion anaemia on the Δchange in cytokines and markers of endothelial activation across the exposures, was limited to the first three transfusions.

In summary, the current data supports the potential for transfusion-related immunomodulation to be present early following preterm birth at a time when the pathophysiologic processes that potentially contribute to the development of the major morbidities associated with extremely preterm birth are likely to be initiated. Further, the recipient response to transfusion exposure appears to be influenced by the number of transfusions they receive. Most importantly, the data suggests that inflammatory responses following transfusion exposure in the extremely preterm infant differ between those receiving

unwashed PRBCs compared to those receiving washed PRBCs. These data add to our growing understanding of TRIM in the extremely preterm infant and highlight the need to further characterise the mechanisms linking PRBC transfusion with increased pro-inflammatory cytokine production and endothelial activation, a biologically plausible link between transfusion exposure and increased incidence of neonatal morbidity and mortality. Ultimately, to determine if the adoption of transfusion with washed PRBCs translates into improved survival free of significant neonatal morbidity in extremely preterm infants requires an adequately powered randomised controlled trial comparing washed with unwashed PRBC transfusion with clinically important outcomes.

Chapter 7

Explorative Analysis of the Influence of Transfusion Donor Gender

7.1 Introduction

It is clear that transfusion exposure increases the risk of death in critically ill adult patients [49] with the risk of both short and long-term mortality increasing in proportion to total number of transfusions received [49 50]. While historically this has been thought to be explained by co-morbidities in the transfused recipient, a growing body of literature suggests that characteristics of the blood donor impact a recipient's prognosis [228, 305]. In particular, emerging evidence supports a role for female donor sex to negatively influence the recipient's response [198]. As a result, the role of blood donor demographics on transfusion efficacy and safety is an emerging area of interest in transfusion medicine research [306-308]. As discussed previously, morbidity in the transfusion recipient may be the result of adverse physiological responses, aberrant immunological reactions, or perhaps both. Indeed, while there is emerging evidence focusing on donor sex-related differences in haemoglobin increment in the recipient, there is very little data examining the effect of donor sex on other physiological biomarkers, immune function or outcomes assessing transfusion effectiveness and safety [309].

Specifically, female donor sex has been proposed to contribute to adverse outcomes for transfusion recipients. Research to do date has primarily focused on plasma and platelet transfusions. Data from retrospective observational studies suggests significantly greater risks of adverse outcomes, in particular the diagnosis of TRALI [202], following transfusion of plasma or platelets from female donors compared to male donors. However, there is increasing awareness that exposure to PRBCs from a female donor may pose a similar risk [199]. Whether similar effects occur in the preterm infant in response to exposure to blood products from female donors has received little attention. A single retrospective study of extremely preterm infants who received a transfusion of either exclusively male or mixture of male and female donor PRBCs reported significantly higher rates of mortality, significant morbidity and prolonged hospital stay in those infants exposed to any female blood [234]. While the underlying

mechanism linking the sex of the donor to higher rates of adverse outcome remains unknown, HLA antibodies are one potential candidate [202]. Approximately 20% of multiparous women have circulating HLA antibodies [207]. These antibodies are known to stimulate circulating leukocytes, neutrophils and other cytotoxic cells, initiating a cascade of inflammatory events and widespread inflammation [205, 210].

The aim of this chapter is to investigate whether the sex of the donor contributes to immune response to PRBC transfusion exposure in the extremely preterm infant. Specifically, this analysis will explore whether sex of the donor contributes to any of the changes in circulating plasma cytokines observed in the extremely preterm infant following transfusion with unwashed and washed PRBCs.

7.2 Methods

As described in Chapter 6, the number of cytokines and markers of endothelial activation that exhibited significant post-transfusion changes increased with each transfusion exposure with the greatest differences seen after the third transfusion. Therefore, the donor sex for each of the first 3 PRBC packs the infant received was obtained from The Australian Red Cross Blood Service. Briefly, transfusion pack identification numbers were recorded from each pack and sent to the New South Wales branch of the Australian Red Cross. The identification number revealed the characteristics of the pack's donor. For the purpose of this study only the sex of the donor was required. Study groups were defined by the donor sex (those who received PRBCs from only male donors for the first three transfusions or those who received any PRBCs from a female donor) and whether they received unwashed or washed PRBCs. Cytokines and markers of endothelial activation data were therefore analysed as 4 groups: unwashed PRBCs male only donors; unwashed PRBCs any female donor; washed PRBCs male only donors; and washed PRBCs any female donor. While all FFP was obtained from male donors, platelets may

be from either male or female. As data on the donor sex for platelet transfusions the infants received was not available, those infants who received a platelet transfusion before any of the 3 PRBC transfusion exposures were excluded from analysis.

Inflammatory cytokines and markers of endothelial activation were analysed using the methods described in Chapter 3. Briefly, prior to the transfusion, infants had 0.4mL whole blood collected with a post-transfusion sample collected 4-6 hours following transfusion completion. Samples were centrifuged and plasma aliquoted for cytokine and markers of endothelial activation analysis. Circulating cytokine levels for each transfusion sample were analysed using the Human High Sensitivity T-Cell Magnetic Bead panel multiplex ELISA (see 3.4.1 High Sensitivity T-Cell Multiplex Assay). Markers of endothelial activation were measured using human sepsis bead panel multiplex ELISA (see 3.4.2 Neonatal Sepsis Assay).

7.2.1 Statistical Analysis

Demographic data is presented as median (IQR) or N (%). Fisher's Exact Test or Pearson Chi-Square was used for comparison between groups. As the cytokine and endothelial markers were not normally distributed the Kruskal-Wallis test was used to compare differences between pre- and post-transfusion concentrations for the 4 groups with a $p<0.05$ considered statistically significant. Where the pre- post-transfusion change was significant for more than one of the groups, the magnitude of the difference (Δchange) was compared by the Kruskal-Wallis test with Dunn's post hoc test to investigate differences between the individual groups, with a $p<0.01$ used to correct for multiple comparisons. Further, hierarchical multiple regression was used to assess the impact of exposure of the four transfusion exposure groups (unwashed PRBCs male only donors; unwashed PRBCs any female donor; washed PRBCs male only donors; and washed PRBCs any female donor) on the magnitude of the change, after controlling for gestational age, newborn sex, and age at first transfusion.

7.3 Results

Baseline antenatal characteristics are shown in Table 7.1. Groups were defined by type of PRBC transfusion (unwashed versus washed) and donor sex. While there were no significant differences in major clinical characteristics including gestational age, exposure to antenatal inflammation, mode of delivery or birthweight, infants who received more than one transfusion with washed blood from a male donor were more likely to be growth restricted and from a pre-eclamptic pregnancy. No differences in any of the groups was observed with transfusion characteristics. The three transfusion exposures occurred within the first three weeks of postnatal life. Unsurprisingly, as transfusion exposure increased, the number of newborns transfused with only blood from a male donor decreased.

There was no significant difference between the groups in pre-transfusion cytokines prior to any of the three transfusion exposures and the baseline pre-transfusion concentrations of the individual cytokines was not significantly different between the 1st and 3rd transfusion exposure.

Table 7.1 Baseline Antenatal Characteristics

	Unwashed Male	Unwashed any Female	Washed Male	Washed any Female	p
Transfusion 1					
n	40	30	55	14	
Gestation	25 (24-27)	26 (25-27)	26 (25-27)	25 (25-26)	ns
Birth Weight	750 (640-960)	850 (660-968)	830 (680-1010)	845 (624-946)	ns
IUGR	6 (13)	4 (13)	13 (22)	1 (6)	ns
Multiple Birth	14 (31)	8 (26)	16 (27)	1 (6)	ns
Antenatal Steroids					
None	4 (9)	1 (3)	2 (4)	3 (16)	ns
Incomplete	10 (22)	4 (13)	10 (17)	5 (28)	
Complete	31 (69)	26 (84)	46 (79)	10 (56)	
Magnesium Sulphate	28 (62)	20 (65)	40 (68)	12 (67)	ns
Chorioamnionitis	19 (42)	10 (32)	19 (32)	5 (28)	ns
Preeclampsia	1 (2)	1 (3)	5 (8)	0 (0)	ns
Mode of Delivery					
SVD	19 (42)	10 (32)	18 (31)	8 (44)	ns
LSCS	26 (58)	21 (68)	41 (69)	10 (56)	

Transfusion 2					
n	17	35	31	24	
Gestation	25 (23-26)	25 (24-27)	26 (24-27)	26 (25-26)	ns
Birth Weight	735 (645-928)	745 (630-890)	790 (660-980)	770 (610-946)	ns
IUGR	1 (5)	5 (14)	10 (29) *	2 (7)	0.05
Multiple Birth	5 (25)	8 (22)	7 (20)	1 (4)	ns
Antenatal Steroids					
None	2 (10)	2 (6)	2 (6)	3 (11)	ns
Incomplete	4 (20)	6 (16)	6 (17)	6 (22)	
Complete	14 (70)	29 (78)	27 (77)	18 (67)	
Magnesium Sulphate	15 (75)	21 (57)	24 (69)	18 (67)	ns
Chorioamnionitis	11 (55)	14 (38)	13 (37)	9 (33)	ns
Preeclampsia	1 (5)	1 (3)	4 (11)	0 (0)	ns
Mode of Delivery					
SVD	9 (45)	13 (35)	8 (23)	12 (44)	ns
LSCS	11 (55)	24 (65)	27 (77)	15 (56)	
Transfusion 3					
n	10	32	16	28	
Gestation	25 (24-26)	25 (24-26)	25 (23-26)	25 (24-26)	ns
Birth Weight	750 (640-890)	663 (610-800)	740 (620-790)	780 (624-946)	ns
IUGR	0 (0)	5 (15)	6 (35) *	3 (9)	0.04
Multiple Birth	2 (18)	8 (24)	2 (12)	3 (9)	ns
Antenatal Steroids					
None	1 (9)	2 (6)	0 (0)	4 (12)	ns
Incomplete	2 (18)	7 (20)	5 (29)	7 (21)	
Complete	8 (73)	25 (74)	12 (71)	22 (67)	
Magnesium Sulphate	8 (73)	21 (62)	13 (76)	20 (61)	ns
Chorioamnionitis	7 (64)	14 (41)	4 (24)	14 (42)	ns
Preeclampsia	0 (0)	0 (0)	4 (24) *	0 (0)	<0.001
Mode of Delivery					
SVD	5 (45)	15 (44)	5 (29)	14 (42)	ns
LSCS	6 (55)	19 (56)	6 (35)	19 (58)	

Data presented as median (IQR) or n (%)

Following the first transfusion IL-10 decreased in those infants in the washed blood male only donors group (17.1 (3.1-30.5) to 10.6 (3.4-17.1) pg/ml, p=0.02), an effect also seen following the second transfusion exposure (14.4 (3.1-58.3) to 12.7 (5.4-28.6) pg/ml, p=0.022). At the second transfusion exposure IL-8 fell in infants transfused with washed blood male only donors group (350.5 (95.5-484.5) to 130.1 (86.5-233.4) pg/ml, p=0.049) and washed blood any females group (231.5 (64.3-370.6) to 88.6 (32.0-172.3) pg/ml, p=0.017).

Table 7.2 Pre-post third transfusion exposure cytokine concentrations

	Unwashed Male (n=10)		Unwashed any Female (n=32)		Washed Male (n=16)		Washed any Female (n=28)	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
IFNγ	11.2 (2.6-26.4)	3.4 (2.1-34.4)	0 (0-15.4)	1.5 (0-34.5)	6.21 (1.1-17.4)	1.54* (0.7-20.6)	4.2 (0.85-21.4)	1* (0.3-6.9)
IL-10	18.3 (4.5-205.2)	8.5 (5.6-55.4)	10.7 (0-22.4)	6.7* (3.4-69.4)	11.8 (1.7-25.1)	4.9* (2.5-11.6)	9.9 (3.1-20.4)	9.6 (6.5-16.0)
IL-12	4.6 (1.8-6.3)	1.2 (0-7.1)	2 (0-6.2)	3.8 (0-15.2)	2.7 (1.7-43.3)	1.5* (1.2-15.3)	6.7 (1.4-9.8)	1.7* (0.8-10.5)
IL-17A	9.8 (5.6-13.9)	7.4 (3.1-14.4)	7.4 (0-18.8)	12.1** (5.6-28.1)	5.8 (2.3-31.7)	1.3* (0.8-15.1)	9.5 (0.8-20.1)	4 (0.4-14.5)
IL-1β	0.42 (0-1.71)	0.9 (0.23-2.9)	0 (0-5.6)	0.7 (0-12.6)	8.1 (0.2-30.5)	0.42 (0.3-5.6)	1 (0.3-4.4)	1.3 (0.3-3.6)
IL-6	10.92 (0-42.3)	18.66 (2.7-38.4)	4.3 (0-29.3)	8.4 (0-50.3)	11.3 (8.7-44.7)	13.9* (6.3-94.8)	20.7 (6.8-39.5)	12.3 (3.6-24.2)
IL-8	207.3 (20.8-640.4)	165.2 (75.2-505.5)	85.8 (35.3-246.2)	134.42 (73.2-256.9)	253.4 (86.5-360)	85.3 (52.8-143.6)	193.9 (52.7-445.8)	118 (44.6-210.1)
TNF	51.1 (31.8-84.9)	37.7 (26-133.4)	45.7 (23.4-66.9)	59** (35.9-107.6)	51.4 (42.3-80)	33.1 (30.5-79)	55.8 (33.7-71.5)	39.7 (26.3-60.2)

Data presented as Median (IQR) pg/mL. *p<0.05 **p=0.01

Table 7.3 ΔChange in plasma cytokine concentration pre- to post third transfusion exposure (pg/ml)

	Unwashed Male (n=10)	Unwashed any Female (n=32)	Washed Male (n=16)	Washed any Female (n=28)	p
IFNγ	-7.7 (-13.3- 0.8)	0 (-0.6- 10.39)	-4.2 (-21.7—0.1)	-1.5 (-6.3—0.1)	0.01
IL-10	-3.8 (-34.7-3.1)	3.4 (0-19.33)	-7.4 (-13.5- -4.4)	-1.6 (-16.2- 2.5)	0.01
IL-12	-1.7 (-1.9- -0.8)	0.4 (0- 5)	-1.5 (-30- -0.7)	-2.3 (-8.2- 0.03)	0.001
IL-17A	0.6 (-1.3- 1.2)	4.8 (-0.6- 12.5)	-4.5 (-21.7- -1)	-1.7 (-10.4- 2.1)	0.002
IL-1β	0 (-0.3- 2.2)	0 (-0.3- 6)	-8.1 (-21.5-0)	-0.1 (-1.4- 1.7)	ns
IL-6	-1.2 (-8.2- 10.8)	0 (-1- 6)	-3.7 (-30- -2.4)	-5.6 (-15.7- -0.2)	0.008
IL-8	-65 (-132.2- 23)	54 (-13.5- 78.3)	-168.1 (-216.4- -33.7)	-40.6 (-258.7- 53.7)	ns
TNF	7 (-3.2- 31.45)	14.5 (-0.8- 43.5)	-13.6 (-18.6- 5.3)	-15.4 (-28.1- 11.9)	ns

Data presented as median (IQR)

The third transfusion exposure was associated with the greatest number of post-transfusion changes in circulating cytokine concentration (Table 7.2). While the 1st and 2nd transfusion exposures only resulted in significant changes in those groups receiving washed blood, the 3rd transfusion exposure also resulted in significant changes in those infants in the unwashed PRBCs any female donor group, with no differences observed in the unwashed PRBCs male only donor group. For those infants receiving washed PRBCs, IFNγ and IL-12 decreased, while IL-6 increased, but only in the washed male donor only group. For the infants receiving unwashed PRBCs, IL-17A and TNF increased. Interestingly, post-transfusion IL-10 concentrations decreased in those newborns transfused with unwashed PRBCs from both males and females and those transfused with washed PRBCs from males alone.

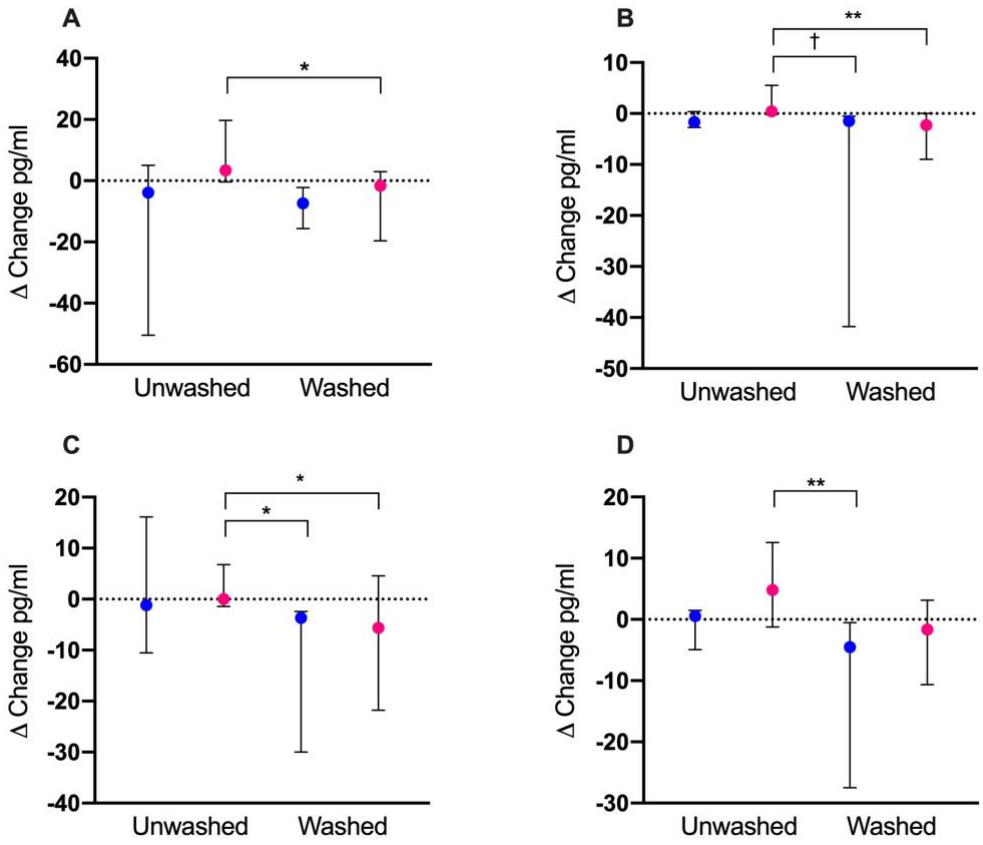

Figure 7.1 Δchange of inflammatory cytokine response to 3rd transfusion exposure (●Male ● Female). A IL-10, B IL-12, C IL-6, D IL-17A, Median (IQR) *p<0.05 **p<0.01 †p<0.001.

To determine if the magnitude of the observed post-transfusion changes in cytokines that occurred in 2 or more of the groups differed the Δchange between the pre- and post-

transfusion concentrations was calculated. A significant difference in the Δchange was seen for IFNγ, IL-6, IL-10, IL-12, and IL-17A (Table 7.3). On post-hoc comparison, the post-transfusion increases in the unwashed PRBCs any female donor group was significantly different from the observed decrease in the washed PRBCs any female donor group (p=0.02) (Figure 7.1A). A similar effect was also seen for IL-12 (p=0.002) (Figure 7.1B) and IL-6 (p=0.02) (Figure 7.1C), with the differences between these two groups for IL-17A close to significance (p=0.07) (Figure 7.1D). Significant differences were also seen between the post-transfusion increases in the unwashed PRBCs any female donor group and decreases in the washed PRBCs male only donor group for IL-12 (p=0.001), IL-6 (p=0.05) and IL-17A (p=0.003).

Where significant post-transfusion differences between pre- to post-transfusion cytokine concentrations observed following the third transfusion, hierarchical multiple regression was used to assess the impact of exposure of the four transfusion exposure groups (unwashed PRBCs male only donors; unwashed PRBCs any female donor; washed PRBCs male only donors; and washed PRBCs any female donor) on the magnitude of the change, after controlling for gestational age, newborn sex, age at first transfusion and degree of pre-transfusion anaemia. Preliminary analyses were conducted to ensure no violation of the assumptions of normality, linearity, multi-collinearity and homoscedasticity. Gestational age, newborn sex, and age at first transfusion were entered at Step 1 with transfusion exposure group entered at Step 2.

For IL-12, gestation, sex, age at first transfusion and degree of pre-transfusion anaemia accounted for 10.6% of the variance in ΔChange in plasma IL-12 concentration. After entry of transfusion exposure group at Step 2 the total variance explained by the model as a whole was 26.2%, $F(5, 55)=4.878$, p=0.002. Transfusion exposure group explained an additional 16% of the variance in ΔChange in IL-12, after controlling for gestation, sex, age at first transfusion and degree of pre-transfusion anaemia, *R* squared change = .16, *F* change (1, 55) = 11.64, p=0.001.

In the final model gestational age and transfusion exposure group were statistically significant, with transfusion exposure group recording a higher β value (β = .41, p=0.001) than gestational age ((β = -.26, p=0.04).

For IL-17A, gestation, sex, age at first transfusion and degree of pre-transfusion anaemia accounted for 14.4% of the variance in ΔChange in plasma IL-17A concentration. After entry of transfusion exposure group at Step 2 the total variance explained by the model as a whole was 29.4%, *F*(4, 54)=5.63, p=0.001. Transfusion exposure group explained an additional 15% of the variance in ΔChange in IL-17A, *R* squared change = .16, *F* change (1, 54) = 11.46, p=0.001. Again, in the final model gestational age and transfusion exposure group were statistically significant, with transfusion exposure group recording a higher β value (β = .4, p=0.001) than gestational age ((β = -.25, p=0.049).

7.4 Discussion

The impact of donor sex on recipient outcome has been demonstrated in a number of areas of transfusion / transplantation medicine. Blood product donor sex is a well-recognised risk factor for TRALI [310, 311], donor-recipient sex mismatch is a recognised risk factor for decreased survival post-cardiac transplant [312], and stem cell recipients show better outcome when cells are donated by men [313]. However, the evidence for an effect of PRBC donor sex on adverse outcomes in the preterm neonatal population has only recently gained attention. A single retrospective observational study has reported an association between exposure to female PRBC transfusion with morbidity and prolonged length of hospital stay [234]. Further analysis comparing exclusively female donor blood with male donor blood demonstrated a dose response effect with a significant interaction of female donor blood and the number of transfusions and morbidity [234].

To date, critically, the biological mechanisms that might explain why donor sex could result in harm in the transfusion recipient remain poorly understood [198]. In the current study we focused on the potential for donor sex to influence TRIM in response to both unwashed and washed PRBCs. The post-transfusion changes in circulating cytokines differed significantly in those infants who received unwashed PRBCs from both male and female donors, compared to those transfused with washed PRBCs. Specifically, by the 3rd transfusion exposure, infants transfused with unwashed PRBCs from both male and female donors had a significant increase in circulating IL-10, IL-12, IL-6 and IL-17A. The opposite was observed for infants transfused with washed PRBCs from only male donors and those transfused with washed PRBCs from both sexes. This data suggests that not only the type of blood, whether unwashed or washed, but also donor characteristics are important. Further, transfusion related changes in immune responses appear central to the recipient's response to PRBC exposure highlighting the complexity of transfusion associated morbidities and mortality.

Some differences between PRBCs from male compared to female donors are fundamental. For instance, PRBCs from male donors are known to have higher total haemoglobin content than those from females, while rates of storage haemolysis are higher [308, 314]. Further, larger increments in haemoglobin concentration are seen in recipients of PRBCs from male donors compared to female donors [308]. In addition, differences in haemoglobin increment in sex-mismatched versus same sex donors have been reported with male recipients of female derived PRBCs having significantly smaller increases in haemoglobin concentration than female recipients of male derived PRBCs [308]. Pre-menopausal women lose blood monthly through menses and therefore their red cell mass is comprised of a higher proportion of young red cells with greater rigidity and better oxygen carrying capacity [307]. Further, a difference in iron status between female and male donors, particularly those female donors who have previously been pregnant, has been proposed to be important, with donor iron deficiency resulting in iron-

deficient erythropoiesis, associated with worse recovery of red blood cells post-transfusion in animal models [315].

Specific attention has been given to a potential role for antibody-mediated immune cell activation by donor HLA-antibodies [310]. HLA molecules are part of the human major histocompatibility system (MHC) and are a family of cell surface glycoproteins that bind to intracellular and extracellular peptides [203]. The HLA family is separated into two distinct functional and structural groups; HLA Class I and HLA Class II [205]. Class I HLA antigens are carried in varying degrees on almost all nucleated cells, whereas Class II antigens are carried on immune cells, such as activated T cells, B cells, macrophages, dendritic cells, cells of the thymus and in certain other cell lineages, such as epithelial and endothelial cells, after exposure to interferon-γ [204]. The overall role of the HLA proteins is induction and regulation of immune responses [205]. However, in the setting of an inappropriate immune response, such as with a sex-mismatched transfusion, the immune response produced by the HLA proteins may be rapid and extremely robust [206].

Anti-HLA antibodies are more common in women than men, even if previously transfused. Importantly, the rates of sensitisation increase significantly in those women with a history of any pregnancy increasing with the total number of pregnancies [316]. The rates of antibody reactivity with MHC Class I and Class II are very similar, ranging from 20-50% detected in those with a history of pregnancy [317]. The most compelling evidence linking anti-HLA antibodies and donor sex to clinical outcomes centres on TRALI where plasma containing blood products from female donors is a well-established risk factor for this major transfusion related morbidity [311]. It has been proposed that HLA class II antibodies activate neutrophils in the pulmonary vasculature by binding to monocytes causing release of pro-inflammatory cytokines and resulting in end-organ damage [218]. Implicated pro-inflammatory cytokines and chemokines

include IFNγ, IL-6 and IL-8 [318, 319], inflammatory mediators which were found to change in a donor sex and PRBC transfusion product specific manner in the current study.

Previously, platelets and plasma products have been the only blood products thought to have a role in the mechanisms by which HLA-antibodies cause an adverse immune reaction [212]. With the surface bound HLA antigen easily detached and shed during storage [205], universal leukodepletion and storage time limits were thought to be the solution to the risk posed by HLA antibodies [207]. However, this is not the case with HLA antibodies detectable in transfused packs [207]. While HLA Class I antigens are carried in varying degrees on erythrocytes [205], the role of PRBCs in antibody-mediated transfusion reactions has been largely overlooked. It has been suggested that a single RBC carries around 100 to 2000 HLA Class I antigens and, as there are 1000 times more red blood cells to leukocytes, the potential for HLA mediated adverse immune responses may, in fact, be more significant than for plasma and platelets [212].

Our observation of significant changes in pro-inflammatory cytokines in relation to exposure to female donor PRBCs after repeat transfusion is consistent with the HLA-antibody hypothesis. Even after leukodepletion HLA antibodies are detectable in transfused packs [207]. In addition, the preterm infant is capable of developing anti-HLA antibodies in response to repeat transfusion exposure [320] despite the assumption that the preterm infant has a functionally immature capacity to secrete immunoglobulins [321]. Finally, washing of transfusion products has been shown to eliminate residual antigens [322-324]. This may explain the potentially beneficial effect seen in those infants receiving blood from female donors, with post-transfusion pro-inflammatory cytokine concentrations significantly lower in those transfused with washed PRBCs compared to those receiving unwashed PRBCs. It is also likely that the relative exposure to plasma elements after a 15ml/kg PRBC transfusion in the extremely preterm infant is greater

than that for a 1-2 unit PRBC transfusion in an adult [234], an exposure that would be reduced following PRBC washing.

The current data represents an exploratory analysis focusing on potential causal pathways underlying the association between PRBC transfusion related adverse outcomes and donor sex. As such, clinical outcomes are not reported. Murphy and colleagues have recently reported a large retrospective observational study on the impact of donor sex on clinical outcomes in a population of preterm infants of similar gestational age. In addition to demonstrating an increased incidence of BPD, NEC and death or any morbidity in those infants who received any PRBC transfusion from a female donor, the odds ratio for any morbidity increased for each additional female donor transfusion [234]. Our finding of greater transfusion related changes in immune function with increasing transfusion exposure would seem consistent with this later finding. There are, however, some important differences between the studies. Obviously, Murphy et al did not investigate the additional contribution of transfusion with unwashed versus washed blood with all PRBC transfusions leukodepleted PRBCs although transfusion volume and duration were similar. In addition, we chose to investigate alterations in circulating pro-inflammatory cytokines in infants who received up to 3 PRBC transfusions. On the contrary, Murphy at el did not control for transfusion exposure and as a result, a significantly higher number of transfusions was received by those in the "female donor" group. This is a potential source of bias with the association between the treatment and the outcome both impacted by the indication. Unfortunately, this is well recognised in transfusion studies and only partially controlled for by attempts to compare illness severity scores between groups. Finally, the studies differ with regard to exposure to other transfusion products. In the current study, we excluded from analysis any infant who received platelets prior to any PRBC transfusion exposure, although we did include infant who had FFP which in our institution is derived solely from male donors. This was principally due to the sex of the platelet donor being unknown due

to the use of pooled platelet units in some. Conversely, Murphy at el excluded infants who received both platelets and FFP.

An important strength of the current study was our approach to classifying donor exposure. As transfusion exposure increases the likelihood of receiving a mixture of male donor only and sex-mis-matched PRBC transfusions increases making it increasingly difficult to define exposure status. By limiting the current study to the first three PRBC transfusions and excluding those who received platelet transfusions we were able to define a simple binary variable (at least one transfusion that was mis-matched versus exclusively male donor only). However, there a number of limitations. The aim of the current study was to determine the effect of exposure to PRBCs from a female donor on immune responses in the transfusion recipient. Unfortunately, due to our sample size, we were unable to recruit a group of extremely preterm, female infants transfused with exclusively female donor blood. Further, we did not investigate the effect of sex mismatched transfusions in females, that is female transfusion recipients transfused with PRBCs from male donors. However, adult data reports a significant increase in the hazard ratio for death for male recipients receiving PRBCs from both female donors with and without a history of pregnancy, while no significant effect was seen in female recipients receiving either sex-matched PRBCs from donor females or sex-mismatched PRBCs from males [228, 233]. Due to limitations in the data collected at the time of blood product donation, we also have no information of the pregnancy status of the female donors nor the HLA status or previous transfusion history for donors of either sex. Finally, as per Chapter 6, post-transfusion concentrations of cytokines were measured at a single time point; therefore, it is not known whether these changes were sustained or transient.

The results of this study highlight the complexity of transfusion responses in a high risk, heavily transfused population. While an area of increasing interest in adults, studies focussing on the

preterm infant remain rare. The current data emphasises the importance of specifically investigating the role of donor characteristics, and particularly sex, in this relatively unexplored area of transfusion research. However, the clinical implications are potentially wide ranging particularly if borne out in follow up studies. If future studies confirm the adverse impact of female sex of donor this would impact not only blood banking processes but also the general donor pool. While the current data suggests that donor sex influences TRIM in the extremely preterm infant, clearly the mechanisms that contribute to these changes remain incompletely characterised. Further, there remains a disconnect between studies with an emphasis on major clinical outcomes and those with a mechanistic focus. As we do not know the sex of the donor when PRBCs are requested for transfusion current practice results in quasi-randomisation of donor sex exposure. However, this apparently random allocation of PRBCs is not a replacement for a randomised trial, one specifically designed to conclusively address the impact of donor sex on both underlying pathophysiologic pathways and clinically relevant outcomes. Clearly, this represents a significant knowledge gap. Any future randomised trial of unwashed versus washed PRBCs transfusion in the extremely preterm infant warrants consideration of donor sex in the study design. It will be also important for future studies that detailed records of the clinical variables of donors as well as recipients are kept.

Chapter 8

General Discussion

8.1 Final Discussion

A tailored approach to PRBC transfusion in the extremely preterm newborn would represent a significant step forward in perinatal medicine. We have reached a point where decades of studies in this heavily transfused population have left the field in an on-going state of uncertainty with recent international surveys confirming that, with respect to transfusion practices, widespread clinical variation continues [325]. The current data provides important foundational information upon which on-going research into the consequences of PRBC transfusion in the preterm newborn can be built. It is clear that allogeneic leukodepleted PRBCs are not an inert product, rather they are biologically active. It is clear that the extremely preterm newborn presents unique challenges. From the current data it could be concluded that exposure to PRBCs represents one of the commonest iatrogenic inflammatory signals in the preterm newborn. If this is the case, a logical response would be to avoid PRBC transfusion if at all possible. It is therefore unsurprising that a fundamental unanswered question in this high-risk group is when to transfuse in addition to what to transfuse with to reduce the risk of unwanted adverse transfusion related complications [326].

Specifically addressing the first question, the relatively small number of high-quality randomised trials comparing liberal versus restrictive transfusion thresholds is a significant problem [136]. While RCTs are generally accepted to be the highest level of evidence it is not infrequent, particularly in perinatal trials, that the available evidence to be derived from trials are too limited in sample size to accurately and therefore convincingly profile efficacy and particularly risk [327]. It is anticipated that upcoming publications of the TOP [328] and ETTNO [329] large-scale randomised trials of transfusion thresholds to guide practice in the preterm newborn may finally provide clarity on when to transfuse. In particular, it is hoped that, when synthesised with the existing evidence which is now over 10-15 years old, there will no longer

be any ambiguity over the effect of liberal versus restrictive transfusion thresholds on death or neurodevelopmental impairment in the extremely preterm newborn.

Are we any closer to accurately reporting the risks associated with transfusion? This is a critical first step for subsequent studies investigating approaches to transfusion that might ameliorate adverse consequence of PRBC exposure. To date, there has been a reliance on observational data as most of the risks associated with transfusion are rare and therefore impossible to accurately evaluate in trials with small sample sizes [265]. Even meta-analysis is inconclusive as potential transfusion-related risks are invariably secondary outcomes with trial based data therefore inconclusive due to lack of power [136]. Indeed, in a recent systematic review focusing on potential adverse outcomes associated with transfusion exposure, in the third of the studies which were randomised the sample sizes were considered inadequate to thoroughly address harm [60]. Further, the authors reported that failure to control for potential confounding factors such as indication bias or a lack of clear, consistent definitions of the different adverse effects related to RBC transfusion in the nonrandomised studies was a recurrent finding [60].

As is the case for defining the correct transfusion threshold, several studies are currently underway which may provide more information on the proposed link between transfusion exposure and neonatal morbidity. These include large observational studies investigating the link between transfusion and ROP [330, 331] and a large multi-national randomised trial investigating the link between PRBC transfusion exposure and NEC specifically focusing on the contribution of withholding feeding prior to, during and immediately after transfusion [236]. What remains a pressing need, as highlighted in this thesis, are clinical trials that incorporate a mechanistic focus.

Physiological Response to Transfusion

With a sample size of 154 extremely preterm newborns, the aims of the current thesis were restricted to potential mechanistic explanations for the association between transfusion exposure with unwashed versus washed PRBCs rather than the impact on clinical outcomes. However, physiological responses to transfusion were investigated with a focus on peri- and post-transfusion changes in cardio-respiratory variables. While changes over time and in response to the type of PRBCs transfused were observed, none met the current agreed criteria for the accepted definitions for either TRALI or TACO employed in other critically ill populations. What does this finding of no transfusion-related physiologic instability mean for this population? One possibility is that clinical correlates of TRALI and TACO do not exist in this high-risk group. However, this seems counter-intuitive given that the volume of each transfusion received by the extremely preterm newborn comparatively far exceeds that in an older child or adult in which both these conditions, although rare, definitely exist. Another possibility is that given the degree of physiological instability common in this group early in the neonatal course, the period in which exposure to PRBCs is greatest, any additional adverse effects on cardio-respiratory function are obscured by the biological noise of extreme prematurity. Finally, the unique nature of the preterm infant's cardiovascular system may cope far superiorly to those of adults and older children.

There was some evidence of inter-play between transfusion associated immune responses and alterations in cardio-respiratory function. Specifically, an association between post-transfusion IL-6 concentrations and decreased systemic vascular resistance and increased cardiac output was seen suggesting a compensatory response to maintain blood pressure and end-organ perfusion in the setting of an increase in pro-inflammatory mediators. However, it is clear that the interaction between physiological and immune responses to transfusion remains to be fully characterised. Indeed, it is likely that the relative contribution of each varies between individual

newborns, influenced by degree of anaemia, illness severity at time of transfusion, and inherent characteristics of the PRBCs transfused. It is also important, when considering the potential for interaction between immune and physiological responses, to consider observations from numerous studies that suggest immunomodulation appears to be "greatest" following exposure to 3 or 4 PRBC transfusions [49, 50, 166, 168]. It is important to highlight that the relationship between physiological responses to transfusion and transfusion related changes in circulating cytokines was investigated for the first exposure to PRBCs in transfusion naïve newborns alone. The possibility that the magnitude of any physiological response to transfusion increases with repeat transfusion exposure, if TRIM is indeed a significant contributor to the underlying pathophysiologic processes, warrants further investigation.

Immune Responses to PRBC Transfusion and are Washed PRBCs the Answer?

The principle focus of this thesis was transfusion-related alterations in immune responses focusing on differences in recipient reactions to unwashed and washed PRBCs. However, exactly how TRIM manifests itself in the extremely preterm newborn is poorly characterised. Indeed, the original observations of decreased helper T-cell count, decreased helper/suppressor T-lymphocyte ratio, decreased natural killer (NK) cell function, defective antigen presentation, decreased cytokine (IL-2, interferon-γ) production, and decreased monocyte/macrophage phagocytic function, led to TRIM being thought of as immunosuppressive [332]. However, a greater awareness that transfusion-related tissue injury is mediated by reactive oxygen species and proteolytic enzymes released from activated neutrophils with endothelial activation highlights a pro-inflammatory component [333]. As a result, it has been proposed that the reported association between PRBC transfusion exposure and *short-term* morbidity and mortality more likely reflects a ''pro-inflammatory'' rather than an ''immunomodulatory'' effect [100].

Numerous pre-clinical studies demonstrate that RBC products directly modulate immune cell function [59]. This has led to the concept of TRIM as "two-insult" process [97] in the transfusion recipient mediated by biological response mediators, such as donor antibodies, bioactive lipids, free haemoglobin, red cell membrane fragments and cytokines that accumulate during blood product storage [95]. This has led to a focus on modifying the transfusion product through leukodepletion and more recently washing [173]. However, research into the effect of RBCs on immune cell function is still preliminary. The current data suggests that not only is the potential for transfusion-related effects of immune function present in the initial days following extremely preterm birth, but changes with ongoing repeated transfusion exposure. Critically, these changes are different in response to transfusion with unwashed versus washed PRBCs. As a result, the use of washed PRBCs could reduce the adverse impact of TRIM, whether this translates to improved clinical outcomes remains unknown and therefore warrant further investigation. Further work needs to be done to accurately classify TRIM in the preterm newborn with evidence from the adult literature suggesting the effects vary between different critically ill populations [100].

In the current study, as for the majority of previous pre-clinical and clinical studies investigating the immune response to transfusion, the cellular components of the blood are removed with the analysis of the responses focusing on bioactive mediators in plasma or serum. While valuable, emerging evidence indicates this represents an incomplete profile of the relevant markers. Until recently red blood cells have been relatively understudied and thought to only be important for respiratory gas exchange. However, recent data suggests they are multifunctional and have more complex functions than previously understood [334, 335]. As a result, red blood cells like other blood products should be considered a complex, living tissue capable of signalling or receiving signals from other cell types with the potential to elicit or participate in the immune response after transfusion. This highlights the importance of

developing a better understanding of TRIM, which may be even more incomplete than previously thought.

Do these new insights contribute to the potentially beneficial effects of transfusion of washed PRBCs on recipient immune function in the extremely preterm newborn? Darbonne and colleagues described a receptor on the surface of red blood cells that binds IL-8 [336]. This was subsequently found to also bind C-X-C (GRO-α, NAP-2) and CeC (RANTES, MCP-1) chemokines [337]. Later identified as Duffy Antigen Receptor for Chemokines (DARC), this receptor was originally identified in 1950 [338] and is known to also be present on endothelial cells [339]. DARC is involved with both binding and releasing cytokines and as a result the RBC has been proposed to act both as a sink for excess circulating inflammatory markers [336, 340] while also acting as a reservoir of these cytokines to release when they are needed [341]. This is an important function, as previously, increases in secreted cytokines from stored RBCs were attributed to WBCs. Intriguingly, washing RBCs significantly attenuates the release of a number of pro-inflammatory cytokines including IL-1β, IL-6, IL-8, MIP-1α, MIP-1β, and TNF [342]. As this data is consistent with our observations of the effect of transfusion with washed PRBCs on post-transfusion cytokine concentrations, it is tempting to speculate that the washing process results in the release of RBC bound cytokines prior to transfusion resulting in reduced capacity to release cytokines and potentially contributing to the altered inflammatory response in the recipient. It would also be consistent with our observation of lower cytokine concentrations in the supernatant of the washed compared to the unwashed transfusion packs. To our knowledge, no detailed analysis of cytokine and chemokine concentrations in the lysate and/or cytosol of washed leukodepleted PRBCs in comparison to unwashed leukodepleted PRBCs has been conducted. This warrants future investigation.

The processes through which RBC release bound cytokines remain unknown. RBCs are devoid of the classic secretion pathways that are well documented for other cell types due to the lack of organelles. However, other mechanisms have been proposed that may have a role in this process including release of microparticles [336, 343, 344]. Microparticles, produced as RBCs age, stimulate a variety of disease processes including immunomodulation [345] [103]. Although various mechanisms of action have been investigated, such as the activation of antigen presenting cells [103], the cytokine contribution from these microparticles has been largely overlooked [346]. DARC is known to be present on the surface of RBC microparticles [347] and retains its capacity to bind cytokines and chemokines [348]. What is not known is its function in cytokine release. Interestingly, not only are microparticles significantly reduced in paediatric washed PRBC transfusion units but washing also alters the microparticle GPA phenotype [172]. Although a number of inflammatory proteins have been identified on RBCs the knowledge of the molecular processes behind the binding and release of many of these cytokines from RBCs remains superficial. Specifically, if washing also alters the expression of DARC on microparticles, does this simple processing step also alter the immunomodulatory effect of microparticles in the transfusion recipient?

The interaction between pro-inflammatory cytokines and the endothelium may also play an important role linking transfusion exposure to adverse clinical outcomes. By the 3rd transfusion exposure, the pro-inflammatory response to unwashed blood was also associated with an increase in markers of endothelial activation, specifically MIF and PAI1, an effect not seen with washed PRBCs. Clearly, inflammatory stimuli directly impact on the endothelium with pro-inflammatory cytokines such as TNF and IL-1β activating endothelial cells and resulting in the recruitment of leukocytes to sites of cellular damage [278]. Central to these processes is the increased expression of several adhesion molecules and chemokines, including MIF, which promotes a number of responses such as the expression of endothelial P-selectin and the arrest

of leukocyte rolling [280]. Known to be produced by a wide range of endothelial cells including both the vascular [349] and pulmonary endothelium [350], MIF has been shown to play a critical role in a wide range of inflammatory conditions [335]. As a result, we chose to investigate the effects of exposure to unwashed and washed PRBCs on this marker of endothelial function. However, it has recently become apparent that RBCs are actually the largest reservoir for this protein in blood, contributing 25μg of this protein per millilitre, a level 1000-fold higher than the average levels detected in plasma [335]. Secreted or free MIF has a primary role as a pro-inflammatory cytokine which modulates the migration of inflammatory cells [351]. However, MIF may also be released following RBC haemolysis, an effect proposed to contribute to the significantly elevated concentrations observed in the plasma of septic patients where high levels of this protein have been correlated with early death [352]. This likely explains the higher levels of MIF in the supernatant of the washed packs with washing known to increase the degree of haemolysis in PRBC pedi-packs up to 14 days of storage, although levels remain well below safety limits [254]. It does, however, highlight the complexity of the interplay between the transfused blood product and the transfusion recipient.

The Contribution of Donor Characteristics to Transfusion-Related Adverse Outcomes

The impact of donor sex on transfusion recipient outcomes has been demonstrated in a number of areas of transfusion / transplantation medicine. However, the evidence for an effect of PRBC donor sex on adverse outcomes in the preterm neonatal population has only recently gained attention. Further, despite increasing attention, the biological mechanisms that might explain why donor sex could cause harm in the transfusion recipient remain poorly understood [198]. The current data demonstrates that the post-transfusion changes in circulating cytokines differ significantly in those newborns who received unwashed PRBCs from both male and female donors compared to those transfused with washed PRBCs. In light of the epidemiological data from the study by Murphy et al which reported higher rates of mortality,

significant morbidity and prolonged hospital stay in those infants exposed to any female blood [234], these findings have significant consequences for both the neonatal and transfusion communities. Further, if these findings are confirmed, likely in the setting of a large-scale randomised trial, they broaden the potential of alterations in blood banking policy to reduce the risk of transfusion associated adverse outcomes in the extremely preterm newborn. Rather than limiting available PRBCs to those solely from male donors, a restriction with wide ranging implications for blood product availability, PRBC washing may represent a single, simple intervention which reduces transfusion associated morbidity and mortality. Clearly, these findings may have significant consequences for transfusion studies already underway and those in the planning stage. The significant impact of donor sex also has implications for other transfusion product exposures. This is particularly important for those pooled products derived from multiple donors such as FFP and particularly platelets. This question was out of the scope of the current thesis and warrants more in-depth investigation.

Future Directions and Translation into Clinical Practice

Given the current data, what are the implications for clinical practice in the field of perinatal medicine? While it is hoped we are about to finally answer the question, "when to transfuse", many more questions remain unanswered. In light of our findings of differing effects on circulating pro-inflammatory cytokines with increasing transfusion exposure, the impact of total volume or number of PRBC transfusion events an individual extremely preterm newborn is exposed to requires attention. For instance, the current data may suggest that fewer, larger volume transfusions may result in a differed inflammatory response following transfusion. Further, an extremely preterm newborn in the first days or weeks of life is likely to be very different from one further along the neonatal journey. If we assume the inflammatory milieu of prematurity is most complex and potentially harmful soon after birth, should all early transfusions be not only washed but also from only male donors? Outside this high-risk period,

could PRBCs from either male or female donors be used or even unwashed leukodepleted PRBCs? Other important considerations which may impact upon future trials include the role of irradiation, transfusion of O negative versus cross match blood and other novel processing steps for instance supernatant reduction [353]. All add to the complexity of clinical trial design in the field of transfusion medicine.

While not designed to demonstrate a clinical advantage of transfusion of washed compared with unwashed PRBCs, the observation of significantly different inflammatory responses in the washed group represents a biologically plausible mechanism by which transfusion with washed PRBCs may be beneficial. Further, epidemiological and observational evidence supports an association between transfusion of washed RBCs and fewer post-transfusion complications in cardiac patients, improved survival in acute leukaemia patients [354] and reduced transfusion-related pro-inflammatory cytokine production in paediatric cardiac surgical patients [355]. Significantly, the potential for clinical benefit in the extremely preterm newborn remains a significant knowledge gap.

Due to the number of transfusions a preterm newborn is exposed to during their primary hospital admission, any improvement in outcome related to a modification in transfusion practice is rapidly translated into clinical practice. To date, the only intervention associated with a clinically important reduction in morbidity in the preterm newborn in relation to PRBC transfusion practice, was the introduction of pre-storage leukodepletion [129]. This led to adoption of this simple processing step world-wide. The current data adds to the growing *in vitro* and *ex vivo* mechanistic literature supporting a different inflammatory response to washed PRBCs compared to unwashed PRBCs. However, an adequately powered randomised trial with a meaningful clinical outcome is the critical next step in order to prove or disprove

whether this intervention has the potential to result in a similar wholesale change in transfusion practice, to achieve improvements in survival free of significant neonatal morbidity.

PRBC washing is already a readily available, standardised process provided by The Australian Red Cross. However, due to greater manufacturing costs incurred with the washing process ($426.94 for a washed leukodepleted RBC pack versus $399.25 for an equivalent unwashed leukodepleted RBC pack) and more importantly shortened shelf life, unwashed leukodepleted PRBCs remain the product of choice in Australia. The data in this thesis not only establishes the clinical feasibility of a randomised trial of unwashed versus washed PRBCs in the NICU environment but provides hypothesis generating evidence focusing on the pathophysiologic processes that may explain the beneficial effect of exposure to washed PRBCs if a transfusion is clinically indicated.

A randomised controlled trial adequately powered with clinically important outcomes and associated health economic outcomes is the only way to determine whether washed PRBCs compared to unwashed PRBCs improve clinical outcomes in extremely preterm newborns. Such an approach would effectively bridge the gap between research into transfusion-related adverse outcomes and translation into practice with clinical and economic evaluation. The current economic burden placed on the individual and the healthcare system of each surviving extremely preterm newborn is immense. For instance, depending on BPD severity, surviving newborns may have a life-time projected economic impact of over $1.2 million AUD [356]. With an additional $30 manufacturing cost for washed PRBCs, even taking into consideration the potential for increased overall wastage of this critical resource due to reduced shelf life, the avoidance of even a single case of significant long-term neonatal morbidity would make this added cost inconsequential.

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Appendices

Appendix 1: Clinical Characteristics of Infants Consented but not Transfused

Table 1. Antenatal characteristics of babies consented but not transfused	
Gestation, weeks	28 (25 – 29)
Male	35 (61)
Birthweight, grams	1060 (446 – 1625)
IUGR	4 (7)
Multiple Birth	17 (30)
Antenatal Steroids	
None	2 (4)
Incomplete	2 (4)
Complete	42 (79)
Repeat	7 (13)
Chorioamnionitis	8 (14)

Data presented as median (minimum – maximum) or n (%)

Table 2. Neonatal characteristics of babies consented but not transfused	
Spontaneous Intestinal Perforation	0 (0)
Necrotising Enterocolitis	2 (4)
Periventricular Leukomalacia	0 (0)
Interventricular Haemorrhage	3 (5)
Retinopathy of Prematurity	0 (0)
Bronchopulmonary Dysplasia	16 (28)
Blood culture positive sepsis	4 (7)
Length of Ventilation	1 (0 – 9)
Length of Nursery Stay	59 (4 – 112)
Death	2 (4)

Data presented as median (minimum – maximum) or n (%)

Appendix 2: Participant Information Sheet and Consent form (Women's and Children's Hospital Participants)

WOMEN'S & CHILDREN'S HEALTH NETWORK
HUMAN RESEARCH ETHICS COMMITTEE (HREC)

Participant Information

Lay Title:

Does washing blood for transfusion make a difference to pre-term babies?

Scientific Title:

Effect of Pre-Transfusion Washing of Red Blood Cells on Neonatal Outcome: A Randomised Controlled Trial

Investigators

A/Prof Michael Stark, & Dr Chad Andersen (Department of Neonatal Medicine, Women's and Children's Hospital), Dr Ben Saxon (Australian Red Cross Blood Transfusion Service & Department of Hematology, Women's and Children's Hospital) Dr Denese Marks (Australian Red Cross Blood Transfusion Service)

Introduction

You are invited to take part in this research project because you are the parent / guardian of a preterm baby who may require a blood transfusion.

Preterm babies often receive a blood transfusion (where blood is administered because the blood count falls) during their stay in the nursery. This is partly because of the small amounts of blood normally taken for monitoring but also because preterm babies make new red blood cells slower. As a result of these factors, the majority of very preterm babies receive a blood transfusion.

The blood used for transfusion is extensively screened by the Australian Red Blood Cross service and is very safe. Washing of blood for transfusion is normally done by the Red Cross for a variety of conditions, is a standard approved blood product and is safe. In sick adults and children research suggests washing may reduce illness. If this benefit applies to very preterm babies it would make a big difference.

What is the purpose of this research project?

It is the aim of this study to work out if washing blood for transfusion makes a difference in preterm babies.

The study will investigate how the washed blood affects problems related to preterm birth but also the type and pattern of very small proteins in the blood stream. These very small proteins act as messengers and are a way of measuring the baby's response to transfusion.

What Does the Study Involve?

Babies born less than 29 weeks will be eligible if the nursery staff caring for your baby decide a red blood cell transfusion is needed.
Babies will have a 50 / 50 chance of receiving either standard blood from the Red Cross or an identical pack of washed blood from the Red Cross. Blood will be prepared in a similar way if further transfusions are required.

In addition, a small blood sample (approximately 6 drops) will be collected before and 2-4 hours after the transfusion. Every effort will be made to collect these blood samples at the same time as routine blood tests ordered by the nursery staff. The specimens will be stored in a special freezer and thawed at a later date to be analysed. Every effort will be made to use the morning blood specimen which is usually discarded after measurement.

Apart from this additional test, your baby's participation in this study will not result in any other change. Your baby will have data collected from their bedside chart and medical history including any clinical problems diagnosed during their stay in the neonatal nursery. None of this information will identify you or your baby in any way.

What are the possible benefits?

We do not expect that your baby will receive any additional benefit from this research. Rather, the results of the study may be important in helping us to look after preterm babies who require a blood transfusion in the future.

What are the possible risks?

The study involves taking a small (6 drops) additional amount of blood from your baby. Whenever possible this will be done at the same time as blood tests ordered by the nursery staff caring for your baby. Further, sucrose will be used to reduce the pain for your baby should an extra heel prick be needed to collect the blood sample. Washed blood for transfusion is a standard, clinically approved blood product for preterm babies and its use does not present any additional risks.

Do I have to take part in this research project?

No, your baby's participation in this research project is voluntary. If you do not wish your baby to take part they don't have to. If you decide your baby can take part and later change your mind, you are free to withdraw your baby from the project at any stage. This will not change your relationship with the medical or nursing teams involved in your babies care.

What if I withdraw from this project?

If you decide to withdraw, please notify a member of the research team. If you decide to leave the project, the researchers will ask you if personal and health information that has already been collected can be kept. Please inform the research team if you would like this information discarded.

How will I be informed of the results of this research project?

The results of this research project will be submitted for publication in a medical journal. This will be made available to the public. A plain language summary of group results will also be made available to you at the end of the study if you request it.

What will happen to information about my baby and I?

The information obtained from you and your baby will be stored under a study number and will be non-identifiable to those outside the research team. The information will be retained for a minimum of 30 years. At the end of this period, the hard copy will be shredded, and the electronic data will be deleted in a secure manner. The data will be stored in locked filing cabinets in the Women's and Children's Hospital. Only the principal and co-investigators will have access to this information.

Any information obtained in connection with this research project that can identify you will remain confidential and will only be used for the purpose of this research project. It will only

be disclosed with your permission, except as required by law. In any publication and / or presentation, information will be provided in such a way that you cannot be identified, except with your permission. Information about your baby's participation will be recorded in their health record.

How can I access my baby's information?

In accordance with relevant laws, you have the right to access the information collected and stored by the researchers about you. You also have the right to request that any information, with which you disagree, be corrected. Please contact one of the researchers if you would like to access your information.

Is this research project approved?

The ethical aspects of this research project have been approved by the Human Research and Ethics Committee of the Women's and Children's Hospital – REC2498/9/15. This project will be carried out according to the National Statement on Ethical Conduct in Human Research (2007) produced by the National Health and Medical Research Council of Australia. This statement has been developed to protect the interests of people who agree to participate in human research studies. Further, this project is registered as a clinical trial with www.clinicaltrials.gov and is publically accessible.

What if I Have a Complaint About the Study?

Should you wish to discuss the approval process or have concerns about your rights as a participant in this research, or you have a complaint about the manner in which the research is conducted, it may be given to the researcher, or if an independent person is preferred, Villis Marshall, Director, Office for Research, (08) 8204 6453 or 0466393503, email: Health.SALHNOfficeforResearch@sa.gov.au. Alternatively you may wish to contact local Human Research Ethics Officer, Research Governance Officer, Southern Adelaide Local Health Network, (08) 8204 6453, email: Health.SALHNOfficeforResearch@sa.gov.au.

CHILDREN, YOUTH & WOMEN'S HEALTH SERVICE (CYWHS)
HUMAN RESEARCH ETHICS COMMITTEE (HREC)

CONSENT FORM

Title: Pre-transfusion washing of red blood cells for preterm infants
SCIENTIFIC TITLE: Effect of pre-transfusion washing of red blood cells on neonatal outcome: A randomised controlled trial

I _____hereby consent to my child's involvement in the research project entitled: Pre-transfusion washing of red blood cells for preterm infants

1. The nature and purpose of the research project described on the attached Information Sheet has been explained to me. I understand it and agree to my child taking part.

2. I understand that my child may not directly benefit by taking part in this study.

3. I acknowledge that the possible risks and/or side effects, discomforts and inconveniences, as outlined in the Information Sheet, have been explained to me.

4. I understand that I can withdraw my child from the study at any stage and that this will not affect medical care or any other aspects of my child's relationship with this healthcare service.

5. I understand that there will be no payment to my child for taking part in this study.

6. I have had the opportunity to discuss taking part in this research project with a family member or friend, and/or have had the opportunity to have a family member or friend present whilst the research project was being explained by the researcher.

7. I am aware that I should retain a copy of the Consent Form, when completed, and the Information Sheet.

8. I understand that I am free to withdraw from the research project, without giving any reason, at any time and that my action will not affect the care my child will receive.

9. I agree to the accessing of my child's medical records.

10. I understand that my child's information will be kept confidential as explained in the information sheet except where there is a requirement by law for it to be divulged.

Signed:..... Relationship to Patient:.....

Dated:.....

I certify that I have explained the study to the parent and consider that he/she understands what is involved.

Signed:.....Title: .....

Dated: .....

Appendix 3: Participant Information Sheet and Consent Form (Flinders Medical Centre Participants)

Southern Adelaide Local Health Network (SALHN)

Participant Information

Lay Title:

Does washing blood for transfusion make a difference to pre-term babies?

Scientific Title:

Effect of Pre-Transfusion Washing of Red Blood Cells on Neonatal Outcome: A Randomised Controlled Trial

Site Specific Investigator

Dr Scott Morris (Neonatal Unit, Flinders Medical Centre)

Investigators

A/Prof Michael Stark, & Dr Chad Andersen (Department of Neonatal Medicine, Women's and Children's Hospital), Dr Ben Saxon (Australian Red Cross Blood Transfusion Service & Department of Hematology, Women's and Children's Hospital) Dr Denese Marks (Australian Red Cross Blood Transfusion Service)

Introduction

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The blood used for transfusion is extensively screened by the Australian Red Blood Cross service and is very safe. Washing of blood for transfusion is normally done by the Red Cross for a variety of conditions, is a standard approved blood product and is safe. In sick adults and children research suggests washing may reduce illness. If this benefit applies to very preterm babies it would make a big difference.

What is the purpose of this research project?

It is the aim of this study to work out if washing blood for transfusion makes a difference in preterm babies.

The study will investigate how the washed blood affects problems related to pre term birth but also the type and pattern of very small proteins in the blood stream. These very small proteins act as messengers and are a way of measuring the baby's response to transfusion.

What Does the Study Involve?

Babies born less than 28^{+6} weeks will be eligible if the nursery staff caring for your baby decide a red blood cell transfusion is needed.

Babies will have a 50 / 50 chance of receiving either standard blood from the Red Cross or an identical pack of washed blood from the Red Cross. Blood will be prepared in a similar way if further transfusions are required.

In addition, a small blood sample (approximately 6 drops) will be collected before and 2-4 hours after the transfusion. Every effort will be made to collect these blood samples at the same time as routine blood tests ordered by the nursery staff. The specimens will be stored in a special freezer and thawed at a later date to be analysed. Every effort will be made to use the morning blood specimen which is usually discarded after measurement.

Apart from this additional test, your baby's participation in this study will not result in any other change. Your baby will have data collected from their bedside chart and medical history including any clinical problems diagnosed during their stay in the neonatal nursery. None of this information will identify you or your baby in any way.

What are the possible benefits?

We do not expect that your baby will receive any additional benefit from this research. Rather, the results of the study may be important in helping us to look after preterm babies who require a blood transfusion in the future.

What are the possible risks?

The study involves taking a small (6 drops) additional amount of blood from your baby. Whenever possible this will be done at the same time as blood tests ordered by the nursery staff caring for your baby. Further, sucrose will be used to reduce the pain for your baby should an extra heel prick be needed to collect the blood sample. Washed blood for transfusion is a standard, clinically approved blood product for preterm babies and its use does not present any additional risks.

Do I have to take part in this research project?

No, your baby's participation in this research project is voluntary. If you do not wish your baby to take part they don't have to. If you decide your baby can take part and later change your mind, you are free to withdraw your baby from the project at any stage. This will not change your relationship with the medical or nursing teams involved in your babies care.

What if I withdraw from this project?

If you decide to withdraw, please notify a member of the research team. If you decide to leave the project, the researchers will ask you if personal and health information that has already been collected can be kept. Please inform the research team if you would like this information discarded.

How will I be informed of the results of this research project?

The results of this research project will be submitted for publication in a medical journal. This will be made available to the public. A plain language summary of group results will also be made available to you at the end of the study if you request it.

What will happen to information about my baby and I?

The information obtained from you and your baby will be stored under a study number and will be non-identifiable to those outside the research team. The information will be retained for a minimum of 30 years. At the end of this period, the hard copy will be shredded and the electronic data will be deleted in a secure manner. The data will be stored in locked filing cabinets in the Women's and Children's Hospital. Only the principal and co-investigators will have access to this information.

Any information obtained in connection with this research project that can identify you will remain confidential and will only be used for the purpose of this research project. It will only be disclosed with your permission, except as required by law. In any publication and / or

presentation, information will be provided in such a way that you cannot be identified, except with your permission. Information about your baby's participation will be recorded in their health record.

How can I access my baby's information?

In accordance with relevant laws, you have the right to access the information collected and stored by the researchers about you. You also have the right to request that any information, with which you disagree, be corrected. Please contact one of the researchers if you would like to access your information.

Is this research project approved?

The ethical aspects of this research project have been approved by the Human Research and Ethics Committee of the Women's and Children's Hospital – REC2498/9/15. This project will be carried out according to the National Statement on Ethical Conduct in Human Research (2007) produced by the National Health and Medical Research Council of Australia. This statement has been developed to protect the interests of people who agree to participate in human research studies. Further, this project is registered as a clinical trial with www.clinicaltrials.gov and is publically accessible.

What if I Have a Complaint About the Study?

Should you wish to discuss the approval process or have concerns about your rights as a participant in this research, or you have a complaint about the manner in which the research is conducted, it may be given to the researcher, or if an independent person is preferred, Villis Marshall, Director, Office for Research, (08) 8204 6453 or 0466393503, email: Health.SALHNOfficeforResearch@sa.gov.au. Alternatively you may wish to contact local Human Research Ethics Officer, Research Governance Officer, Southern Adelaide Local Health Network, (08) 8204 6453, email: Health.SALHNOfficeforResearch@sa.gov.au.

Southern Adelaide Local Health Network (SALHN)
HUMAN RESEARCH ETHICS COMMITTEE (HREC)

CONSENT FORM

Title: Pre-transfusion washing of red blood cells for preterm infants

SCIENTIFIC TITLE: Effect of pre-transfusion washing of red blood cells on neonatal outcome: A randomised controlled trial

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I hereby consent to my child's involvement in the research project entitled: Pre-transfusion washing of red blood cells for preterm infants

1. The nature and purpose of the research project described on the attached Information Sheet has been explained to me. I understand it and agree to my child taking part.

2. I understand that my child may not directly benefit by taking part in this study.

3. I acknowledge that the possible risks and/or side effects, discomforts and inconveniences, as outlined in the Information Sheet, have been explained to me.

4. I understand that I can withdraw my child from the study at any stage and that this will not affect medical care or any other aspects of my child's relationship with this healthcare service.

5. I understand that there will be no payment to my child for taking part in this study.

6. I have had the opportunity to discuss taking part in this research project with a family member or friend, and/or have had the opportunity to have a family member or friend present whilst the research project was being explained by the researcher.

7. I am aware that I should retain a copy of the Consent Form, when completed, and the Information Sheet.

8. I understand that I am free to withdraw from the research project, without giving any reason, at any time and that my action will not affect the care my child will receive.

9. I agree to the accessing of my child's medical records.

10. I understand that my child's information will be kept confidential as explained in the information sheet except where there is a requirement by law for it to be divulged.

Signed:..... Relationship to Patient:.....

Dated:.....

I certify that I have explained the study to the parent and consider that he/she understands what is involved.

Signed:.....Title: .....

Dated: .....

Appendix 4: Ethics Approval

Government of South Australia | Health
Women's and Children's Health Network

Women's & Children's Hospital

Research Secretariat
Women's and Children's Health Network
2nd floor
Samuel Way Building
72 King William Road
NORTH ADELAIDE SA 5006
Tel 08 8161 6521
Tel 08 8161 8175
www.wch.sa.gov.au

13 November 2019

Dr Michael Stark
WABS
QVB

Dear Michael

Re: Effect of Pre-Transfusion Washing of Red Blood Cells on Neonatal Outcome: A Randomised Controlled Trial. HREC/12/WCHN/55. Expiry date 31December 2020

At its meeting on 13 November 2019, the WCHN Human Research Ethics Committee approved your request to extend ethical approval for a further five months (from August to December 2020). Please note the amended approval number above reflecting the extension, and use it in any future communications.

Please ensure that an annual report for the study is provided annually via the WCHN Research Annual Reports email (Health.WCHNResearchAnnualReports@sa.gov.au).

I remind you continued approval is given subject to:
•immediate notification of any serious or unexpected adverse events to participants;
•immediate notification of any unforeseen events that might affect continued ethical acceptability of the project;
•submission of any proposed changes to the original protocol. Changes must be approved by the Committee before they are implemented;
•immediate advice, giving reasons, if the protocol is discontinued before its completion;
•submission of an annual report on the study's progress and a final report on completion to the WCHN Research Governance Officer. It is your responsibility to provide these reports, without reminder from the Committee.

I also remind you of the institution's research governance requirements. If the study involves non WCHN staff or students, a signed Confidentiality Agreement is to be provided to Dr Carmel Murone, Research Governance Officer, WCHN Research Secretariat. Additionally, if they visit any WCHN site or access identifiable patient information, a verified copy of their Department for Communities & Social Inclusion (DCSI) National Criminal History Record Check (Child related employment screening) is to be provided to Dr Murone and the Human Resources Department. The study may continue on this proviso.

Yours sincerely

TAMARA ZUTLEVICS (DR)
CHAIR
WCHN HUMAN RESEARCH ETHICS COMMITTEE

Australian Red Cross
BLOOD SERVICE

12 November 2019

A/Prof Michael Stark
Senior Staff Specialist
Department of Neonatal Medicine
Women's and Children's Hospital Adelaide
Theme Leader (Early Origins of Health)
The Robinson Research Institute
The University of Adelaide

Dear A/Prof Stark,

Reference number: 2013#06
Project title: Effect of pre-transfusion red cell washing on neonatal outcome: A randomised controlled trial

Thank you for submitting the above research project for ethical review. The extension to this project submitted on 12 November 2019 was approved by the Secretary on behalf of the Blood Service Ethics Committee. The ethical approval for this project will now lapse on 31 December 2020.

Please note that all requirements of the original ethical approval for this project still apply. The Blood Service Ethics Committee wishes you every continued success in your research.

Should you require any further information, please contact the Ethics Secretary on 02 9234 2368 or at ethics@redcrossblood.org.au.

Yours faithfully,

Dr Larissa Aldridge
Ethics Secretary
Research and Development
Australian Red Cross Blood Service

The Blood Service Ethics Committee is constituted and operates in accordance with the National Health and Medical Research Council's (NHMRC) *National Statement on Ethical Conduct in Human Research (2007).*

Australian Red Cross Blood Service
17 O'Riordan Street
Alexandria | NSW | 2015
P: +61 2 9234 2368 | F: +61 2 9234 2411
Web: www.donateblood.com.au